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Role of porcine circovirus in postweaning multisystemic wasting syndrome

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Role of porcine circovirus in postweaning multisystemic wasting syndrome

by

Roman Mikhaylovich Pogranichnyy

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
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Has met the thesis requirements of Iowa State University

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Signatures have been redacted for privacy

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ABSTRACT

The role of porcine circovirus type 2 (PCV2) in a newly emerged disease, postweaning multisystemic wasting syndrome (PMWS) was studied using two approaches: experimental inoculation and field-based case-control study. In the animal trial, 5-week-old gnotobiotic pigs free of PCV2 were inoculated intranasally and intramuscularly with PCV2 ISUVDL 98-15237 at a rate of 10^4 TCID₅₀/ml and monitored for a 35-day period. Inoculated pigs were viremic at day 7 post inoculation (PI) and developed virus-specific antibody response which was measurable by indirect fluorescent antibody and serum-virus neutralization tests. Viral DNA and antigens were detected in tissues with subtle histopathological changes (i.e., depletion of lymphocytes) at the end of the study (35 days PI). However, no clinical signs described in pigs affected by PMWS were observed in any of the inoculated animals during the study period. These inconclusive observations prompted a case-control study to assess the strength of association of PCV-2 and some other major swine viruses with PMWS. Cases were pigs affected by PMWS based on clinical and diagnostic criteria, whereas controls were clinically unaffected pigs. The proportion of case and control pigs positive for each virus was assessed and statistically compared for the association strength with PMWS. In addition, PCV2 isolates from 6 cases and 4 controls were selected and genetically compared. Type 2 PCV showed a higher association with PMWS (OR=9.3, 1.9 <95% CI<45.3) than other viruses. Risk for PWMS was much higher if an animal

was co-infected with porcine reproductive and respiratory syndrome virus (OR=31.2, 4.1<95% CI<238). However, PCV2 was also found in controls (35/56) and was not detected in 2 of the 31 PMWS pigs. Furthermore, no significant genetic difference was observed among PCV2 isolates from PMWS and clinically normal pigs. Collectively, naïve swine were shown to be susceptible to PCV2. However, the causal role of PCV2 in PMWS could not be conclusively demonstrated. Development of PMWS may require additional factor(s). Since the virus appeared to be widespread in the U.S. swine population regardless of their clinical status related to PMWS, further work remains to determine the pathogenesis of PCV2 in conjunction with PMWS.

INTRODUCTION

Thesis Organization

This thesis begins with an abstract and is followed by review of the literature and a statement of the problem. Two papers present the experimental work that has been done. The graduate student is the principal investigator and senior author for both papers. This thesis concludes with a general discussion, a list of references cited in the literature review, general discussion, and acknowledgments.

Literature Review

Porcine circovirus

History and taxonomy

Porcine circovirus (PCV) is a small, nonenveloped DNA virus approximately 17 nm in diameter. The virus first was recognized as a noncytopathic viral contaminant of a continuous pig kidney cell line PK-15 (ATCC-CCL31) (102;106). Based on morphology and genomic organization the virus has been classified into the Family *Circoviridae* (46). Other members of this family include chicken anemia virus (CAV) and Psittacine beak and feather disease virus (PBFDV), pigeon circovirus, and human circovirus (46;58). Although all viruses were placed in this family based on the similarity of genomic organization, neither DNA sequence nor antigenic homology have been demonstrated among these viruses (9;108).

In 1998, a DNA viral agent morphologically similar to PCV was isolated from pigs with clinical history of progressive weight and respiratory distress in a "high-health-status" herd in western Canada. However, the new virus is antigenically and

genetically distinct from PCV initially identified in the PK-15 cell line (1;59;60). To reflect such a difference, circoviruses isolated from pigs clinically affected with “wasting syndrome” were designated as PCV type 2 (PCV2) and PK-15 cell line contaminant PCV type 1 (PCV1) (1;59). Although PCV2 was recently recognized, a few retrospective studies presented the evidence that PCV2 was present in the domestic swine population as early as 1973 (111).

Physicochemical properties

Circoviral virions are icosahedral symmetry in shape. The virus contains negative-sense, single-stranded circular DNA (102). The buoyant density of PCV is 1.33 -1.37 g/cm³ on CsCl gradient (7;106). Type 1 PCV does not hemagglutinate erythrocytes of various animal species such as pig, sheep, cattle, chicken, turkey, and guinea . The virus is highly resistant to environmental stress. A study demonstrated that the infectivity of PCV was maintained even after exposure to pH3 and high temperature (56⁰C and 70⁰C) for 15 minutes (7). The PCV is stable to chloroform treatment, which supports that the virus is non-enveloped.

Genomic organization and gene expression

Sequence analysis demonstrated that the genome of PCV1 and PCV2 are 1759 nucleotides (nt)(51) and 1768 nt long, respectively (30;58-60). Types 1 and 2 PCV share less than 80% nucleotide sequence homology (59;60) and approximately 75% amino acid homology (60). Computer-aided analyses of PCV1 and PCV2 DNA have identified 11 potential open reading frames (ORFs) (30). The

ORF1 is more conserved between PCV1 and PCV2 than other ORFs, and PCV2 shares 83% and 86% homology with PCV1 at nucleotide and amino acid levels, respectively (60). In contrast, 67% nucleotide and 65% amino acid homology was observed in ORF2 between PCV1 and PCV2 (60). It was suggested that ORF2 or its product could be used for differentiating the two types of PCV (48). Even less homology in ORFs 5, 6, 9, 10, and 11 was reported between PCV1 and PCV2 (30).

Estimated molecular size of deduced polypeptide products encoded by individual potential ORF ranges from 36 to 2 kilodaltons (kD)(30). Open reading frame 1 was expected to encode for the putative Rep protein, which is required for genome replication (50). Predicted molecular mass of ORF1 product is 35.7 kD for PCV1 and 35,8 kD for PCV2. Open reading frame 2 was postulated to encode for capsid protein, which is a major structure protein (50). Based on linear sequence analysis, molecular mass of ORF2 product was predicted to be 27.8 kD for PCV1 and 27.8 kD for PCV2 (30). The predicted size of proteins encoded by individual ORF 3 to 11 of PCV1 was reported to be approximately 23, 13, 10, 6.7, 6, 4, 3.4, 3.7, and 3 kD, respectively. The molecular mass of PCV2 ORF 3 to 11 products was predicted to be approximately 12, 7, 6, 3, 2, 2, 5, 4, and 2 kD, respectively (30). Although computer-aided sequence analyses identified the presence of 11 potential ORFs, only one protein with molecular mass of 36kD was immunologically and chemically identified in the PCV1 virion (102), suggesting that this polypeptide is the only structural protein. Recently, another protein with molecular mass of 31-33 kD has been identified in PCV1 (99). The actual composition of PCV2 structural

protein(s) has not been characterized; yet a recent study suggested that ORF2 of PCV2 encodes for viral capsid protein (62).

It has been speculated that structural protein arises by splicing and transcription taking place in the nucleus on both DNA strands to generate three distinct PCV PK-15 specific RNA transcripts (59). ORF 2 and ORF 4 are more conserved and are possible candidates to code structural protein (59).

There is some evidence indicating that PCV shares a degree of homology to plant nanoviruses (27;58) and homology with newly identified human TT virus (61), but structural protein has not been well characterized.

Viral replication

Based on tissue immunostaining and virus isolation, PCV has been found to infect many different types of cells in a variety of tissues. The virus or viral antigens were isolated/detected in liver, spleen, Peyer's patches of intestine, lung, tonsil, kidney, and other tissues except the central nervous system (5;81). In these tissues, main target cells are lymphocytes, macrophages, hepatocytes, renal tubular and ileal epithelial cells (81). Intensive basophilic or magenta inclusion bodies of 5-25 μm in size can be seen within cells on H&E stained slides of tissues from pigs with circovirus infection (33;41). However, it is not known how porcine circoviruses establish infection and replicate in pigs.

In vitro PCV can replicate in a continuous pig kidney cell line PK-15, a monkey kidney cell line Vero, semi-continuous pig lung cells, semi-continuous swine testicle cells, primary bovine kidney, semi-continuous bovine lung cells, semi-

continuous bovine testicle cells, primary lamb kidney, semi-continuous lamb testicle cells, and other primary and permanent cell cultures (7). In *in-vitro* cell culture the virus does not cause visible lytic cytopathic changes in cells (7;102). Therefore, the presence of the virus can only be demonstrated by indirect methods such as immunofluorescence assay (IFA) (6;22;54;91;101), and *in situ* hybridization (ISH) (6;13;23;54), and polymerase chain reaction (PCR) based assays (43;60). Using these techniques, PCV can be found in only a small number of cells in different tissues. Cytoplasmic inclusion bodies which consists of electron-dense paracrystalline arrays of small non-enveloped viral particles very heterogeneous are also found in inoculated cells (41;92). *In vitro* circovirus replication depends on the availability of cellular enzymes which are expressed during the S phase of cell growth (105). Yield of progeny virus and the number of infected cells in *in-vitro* culture were found to be increased by pretreatment of cells with 300 mM D-glucosamine-HCl, but care must be taken since the reagent has a toxic effect on the cell culture (6;101). D-glucosamine-HCl synchronizes the cell cycle and initiates virus replication by enabling the PCV genome to enter the cell nucleus. If the cell is not treated, the virus will enter the cell nucleus of the daughter cell at the end of mitosis, resulting in a very few cells infected (105). Early protein(s) and non-structural protein(s) of PCV are involved in induction of viral DNA to replicate and regulate this process (104). Accumulation of structural protein is an independent process from DNA synthesis, which takes place in cytoplasm of infected cells and causes cell destruction, however visible cytopathic effect could be demonstrated on limiting cell layers and colonies in soft agar (104).

It has been proposed that circovirus replicates via a double stranded replicated form (RF) by rolling cycle replication (51;58). The predicted product of ORF 4 encodes a Rep protein of 312 amino acids which shows similarities to replication proteins of other plant circoviruses and geminiviruses (50).

Epidemiology

Prevalence and geographic distribution. The presence of PCV has been reported at least serologically in the USA, Canada, Northern Ireland, France, Spain, Great Britain, Korea, and Germany (6;13;19;21;41;45;88;100). The limited numbers of serological surveys have demonstrated that PCV infection is ubiquitous among the swine population (7;19;100;103;111). In one study, prevalence of PCV infection was estimated to be 95 percent in the German swine population (105). However, early serological surveys were conducted using PCV1 as antigen. Recent studies revealed the cross reactivity between PCV1 and PCV2 (6;48). Consequently, accurate prevalence of PCV1 or PCV2 infection needs to be re-assessed. Recently, using a multiplex polymerase chain reaction assay which allows typing of PCV, it has been demonstrated that PCV2 is the main type of PCV circulating in the swine population (24;43). Only 4-8% of the virus present in the field was PCV1.

Transmission mode. Field observations suggested that PCV can cross the placenta and infect fetuses, i.e., congenital infection (5). PCV2 has been detected in hearts or fetal thoracic fluids of aborted or stillborn fetuses (Janke BH, personal communications). Experimentally, vertical transmission was demonstrated by

inoculating a sow with PCV1 during pregnancy and attempting to isolate the virus from fetuses at term or the litter of pigs right after the birth (35); (Joo HS, personal communication).

Types 1 and 2 PCV were found in nasal secretions and feces from experimentally inoculated pigs although animals did not show any clinical signs or pathological changes (5;42;103). These observations indicate that horizontal transmission may occur.

Pathogenicity and disease

Although early sero-epidemiological surveys demonstrated that PCV1 was prevalent in swine, the virus has been detected by PCR assays in about 4-5% of pigs with no clinical signs or histopathological lesions of PMWS(43;49) and 5.7% of pigs with histopathological lesions typical for PMWS (72). Under experimental conditions, PCV1 was detected in various tissues of inoculated animals and shed in nasal secretions and through feces, suggesting that naïve pigs are susceptible to PCV1 (5;103). These observations also suggest that the virus can be transmitted to pen mates. Horizontal transmission was demonstrated in pigs commingled with inoculated pigs. However, experimental inoculation of PCV1 failed to induce any pathological changes and clinical disease in pigs, suggesting that PCV1 is not pathogenic for swine (42;103). In contrast, tremors were reproduced in young pigs inoculated with PCV1 isolates from newborn piglets clinically affected by congenital tremor, but the virus was not genetically or antigenically characterized (35).

Furthermore, PCV1 was found in aborted fetuses collected from the field in

conjunction with reproductive failure and also in fetuses obtained from pregnant sows inoculated with the virus, suggesting that the virus can cross the placental barrier. However, the significance of these findings is unknown.

Type 2 PCV has been implicated in dermatitis/nephropathy syndrome (2;81), pig tremors (35), and wasting syndrome (22;33). However, a causal role of PCV2 in these diseases has not been conclusively demonstrated under experimental conditions.

Other circoviruses

Chicken anemia virus (CAV)

The virus was first identified in Japan from chickens with clinical signs such as depression, severe anemia and reluctance to move with 50% mortality (116). Clinically CAV infection is characterized by slow growing chicks, depression, ruffled feathers, anemia and marked pallor that extends to internal organs (113). Serological surveys indicated that this virus is ubiquitous in commercial chicken flocks (56).

Chicken anemia virus is a icosahedron and contains 2.3kb single-stranded circular DNA (56;107). The size of the virion is approximately 18-26 nm in diameter and the density is 1.35-1.36 g/ml (57;107). The virus is antigenically and genetically distinct from PCV and psittacine beak and feather disease (BFDV) (9;108). Three ORFs have been identified and encode for 3 putative proteins with molecular weight of 51.7, 24.1, and 13.3 kDa, respectively (67) and only one protein with molecular mass of 50 kD was detected using an electrophoresis system (107).

Chicken anemia virus has been reported to be associated with other diseases or syndromes in chicken, such as blue wing disease, hemorrhagic anemia syndrome, gangrenous dermatitis, and aplastic anemia (53). In severe cases when naïve hens are infected with CAV, infected animals show anemia, subcutaneous hemorrhages and gangrenous dermatitis, which leads to enhanced susceptibility to secondary viral or bacterial infections (82;83). The virus can be detected in all tissues, but thymus and bone marrow are reported to be the most severely damaged (98). Usually infected birds die but some may recover after 3-4 weeks(83).

Experimentally the disease was demonstrated in chicks inoculated with CAV at one day of age but was more difficult to be reproduced in chicks at 3 weeks of age or older or in chickens with neutralizing antibody (114). The pathogenicity of CAV in chickens is reported to be enhanced when concurrently infected with infectious bursa disease virus (IBDV) or Marek's disease virus (MDV) (115). Dual infection results in much higher mortality and morbidity and more severe lesions than CAV infection alone (55).

Histopathological lesions in infected chickens are characterized by lymphocyte depletion in the cortex and medulla of thymus and bone marrow. Infected chickens are also depleted of erythrocytes, thrombocytes and granulocytes, and precursors of these cells are replaced by adipose tissue (83). The bursa of Fabricius and spleen are also depleted of lymphoid cells but less severely than the thymus (83). There is some degree of swelling in the liver and Bursa of Fabricius. Muscular atrophy and hemorrhage has been observed

(98;116). Severe lymphoid depletion in lymphoid tissue and the hematopoietic system lead to the speculation that CAV may induces immunosuppression. In one study, immune response of infected chickens to different vaccines was depressed after inoculation with CAV (71).

Exposing breeder flocks to the virus before chickens go to egg production prevents transovarian transmission. Acquired immunity in the flock is known to prevent from vertical transmission and horizontal transmission for 1 day till 2 wk (83). In the United States, the current control strategy for CAV is to expose serologically negative birds at 12-15 weeks of age to known positive flocks (25). In Europe, vaccination with autogenous live virus vaccine has been proven to prevent vertical transmission of CAV by mimicking natural infection (25). Hens that are hyperimmunized with an inactivated CAV vaccine perform better than unvaccinated hens and have better body weight gain, livability, and feed conversion rate. Furthermore, vaccination was reported to be efficacious in protecting chicks from developing the disease on a farm with ongoing disease problems (25).

Psittacine beak and feather disease virus (PBFDV)

The PBFDV is another member of *Circoviridae* family and a circular single-stranded DNA virus with icosahedral symmetry (80). The virus infects a wide variety of species of wild and captive birds and causes anorexia, vomiting, weakness, dystrophic feathers, deformity of beak and bones. In some cases, severe leukopenia or anemia and pancytopenia have been observed (87).

Histopathologically hepatic, necrosis and atrophy of lymphoid follicles with occasional necrotic foci and typical circoviral basophilic, polymorphic inclusion bodies are frequently observed (87). Depletion of lymphoid tissue and necrosis was reported in naturally exposed birds (73). Necrosis and degeneration of epithelial cells lining the developing feathers follicles are common since the virus infects and replicates in these cells (37;44;78). The virus can also be detected in the thymus and bursa with mild infiltration of the cells and necrosis of severely infected cells in the beak and feather follicle. Necrosis of epithelial cells of the tongue, and mouth has been reported (37;78).

Birds can harbor the virus for 10-15 years once exposed, and most develop cryptosporidial infections that generally occur in birds with immunodeficiency. The majority of infected adults develop viremia; however, birds with good established humoral and cell-mediate immunity clear the virus and undergo asymptomatic infection (79). Several factors are considered to be important as to whether birds will have protective immune response or develop fetal disease. Some of these factors include the presence or absence of maternal antibodies, route of viral exposure, exposure dose, and presence or absence of some conditions where birds become immunotolerant (77).

TT virus

In 1997, a novel DNA initially designated 'N22 sequence' was detected by a representational difference analysis and PCR in sera from patients who developed elevated alanine aminotransferase (ALT) levels and clinical hepatitis some time

after receiving blood transfusions during surgical operations in Japan (65). The patients were negative for known etiological agents of Hepatitis A to G. The N22 sequence was not detected in sera collected from these patients prior to the blood transfusion. A material that contains the N22 sequence was fractionated on sucrose gradient (1.26 g/cm^3) from plasma obtained from a blood donor positive for the N22 sequence. Because of its density, filterability and resistance to DNase digestion, the fractionated material was suspected to be virus particles and named TT virus (TTV) after the initials (T.T.) of the patient from whom the N22 sequence was first detected and cloned (65). After the initial discovery of TTV, the detection of TTV DNA in individuals with hepatitis symptoms has been reported in many other countries, such as Taiwan, Brazil, Egypt, Vietnam, Italy, and USA (11;12;15;26;38;52;63;64;70;74).

To date, no successful isolation of TTV has been made from clinical specimens using *in vitro* cell culture techniques. Based on biophysical characteristics, TTV is believed to be a non-enveloped DNA virus since the infectivity was maintained after treatment with chemicals capable of disrupting the viral envelope (69). The size of the virus was estimated to be 30-50 nm in diameter using a filtration technique (61). The buoyant density of the virus is 1.31-1.34 g/ml in cesium chloride gradient (61).

The genome of TTV is a circular single-stranded, negative-sense DNA with the size of 3,818 – 3,853 bases depending upon genotype (61;69). Genetic analyses identified two putative open reading frames (ORF1 and ORF2) that are flanked by a noncoding region of approximately 1.2 kilobases. The noncoding

region is divided into 2 by a GC rich stem loop structure present in the middle of the region. The ORF1 consists of 2,283 – 2,310 bases depending upon genotype, which encodes for a polypeptide consisting of 761 – 770 amino acids (66;69;97). The N22 sequence is a part of ORF1, which has been shown to be highly conserve among TTV isolates within the same genotype (97). Hypervariable regions of ORF1 were also identified among isolates, particularly those collected during the chronic stage of infection (66). Although the genomic structure of TTV ORF1 is similar to that of CAV replicase gene (ORF1), a recent molecular characterization suggests that ORF1 should encode for the nucleocapsid protein (69). Nonetheless, it is not known whether ORF1 product can be immunologically recognized.

The ORF2 consists of 450 – 609 bases depending upon genotype, and is postulated to encode for a nonstructural protein of 150 – 203 amino acids in length and play a role in virus replication (61;69). A recent *in vitro* translation study demonstrated the ORF2 product with molecular mass of 23 kD (97). However, TTV isolates of some genotypes contain an in-frame stop codon that takes the ORF2 apart (i.e., ORF2a and ORF2b), resulting in a protein product with a molecular mass of 17kD. The ORF2 has been demonstrated to be highly variable among isolates of TTV (97).

Genomic structure, gene expression strategy and biophysical characteristics of TTV are very similar to those of members in the Family *Circoviridae*; yet it is distinctly different from circoviruses. TTV contains the a genome almost twice as large as circoviruses. There are several distinct genotypes reported, demonstrating significant genetic heterogeneity among TTV (95). In addition, no serological cross

reactivity between TTV and circoviruses has been demonstrated. At present, TTV is tentatively classified into the Family *Circonoviridae* (61).

TTV has been reportedly associated with post-transfusion non-A to G hepatitis in humans, although Kock's postulates have not yet been fulfilled. Hospital-based descriptive studies showed an increase and then a decrease in the level of TTV DNA in sera collected over time from patients with post-transfusion hepatitis with unknown etiology and elevated ALT level (74;109). In these patients, development of TTV-specific serum antibody followed the disappearance of TTV DNA from the serum. DNA of TTV has also been detected in peripheral blood mononuclear cells and bone marrow from such patients (40).

Besides accidental transmission of TTV through blood transfusion or blood products in humans (47;61;65), some species of non-human primates were shown to be susceptible to TTV under experimental conditions. Chimpanzees injected with human serum or plasma containing TTV which were collected from patients with non-A to G hepatitis developed viremia (61;68). The TTV genome was detected by PCR as early as 98 days postinoculation. Thereafter, the animals were viremic for as long as 70 days after initial detection. In addition, oro-fecal transmission of TTV was also demonstrated in Rhesus monkeys inoculated orally with the virus extracted from the stool of humans positive for TTV (47). The monkeys developed viremia of TTV. Eventhough virus replication was readily established in inoculated animals, neither abnormal blood chemistry nor histopathological evidence of hepatitis was observed in the inoculated animals (47;61).

Although the TTV genome is frequently found in patients with hepatitis, epidemiological surveys using the PCR technique on randomly selected human subjects suggest that the virus is ubiquitous in the human population (17;18;29;36;74). No difference in the frequency TTV detection or the level of TTV in serum was found between individuals with and without hepatitis or altered ALT levels. Despite the ubiquity of TTV in the human population, a correlation between the TTV infection and any specific disease has not yet been established. Furthermore, no association of virus infection with pathological changes has been identified. Consequently, the etiological role of TTV in hepatitis is still in question (94;96). Clinical significance and pathogenesis of TTV infection remains to be determined.

Postweaning multisystemic wasting syndrome (PMWS)

Clinical manifestation

Postweaning multisystemic wasting syndrome is a newly emerged problem in the swine industry. The syndrome was first recognized in so-called “high health status” swine herds in western part of Canada (14;31) and thereafter, in the United States, Northern Ireland, Spain, South Korea, France, Japan (6;13;22;33;86;88). The syndrome was most commonly observed in growing pigs at 4-16 weeks of age. Cardinal clinical signs commonly observed in affected animals are progressive weight loss, tachypnea, unthriftiness and less commonly, icterus, jaundice and diarrhea. Morbidity typically ranges between 5 and 15% in an infected herd (34;90) but mortality among affected pigs can be up to 80% (34).

Pathological findings

Typical gross lesions in affected animals are lymphadenopathy (e.g., enlargement of inguinal lymph node), icterus, renal petichiae, noncollapsed and mottled lungs, skin pallor, yellowish liver with some atrophy, and enlarged spleen. Intestines are filled with fluid with occasional edema of the wall of the cecum. Stomach ulcers and edema of the gastric wall have been observed (6;14;22).

Microscopically depletion of lymphocytes in secondary lymphoid tissues, interstitial pneumonia, and lymphohistiocytic to granulomatous inflammation in multiple organs, predominantly lungs and lymphoid tissues, liver, kidney, pancreas and intestine, are commonly observed (6;14;22). Characteristic microscopic changes in lymphoid tissues include lymphocyte depletion with pyknosis and karyorrhexis in lymphoid follicles and extensive macrophage infiltration. Lymphoid hypoplasia and macrophage infiltration of cortex and medulla with syncytia formation were also reported (39). Histopathological changes in livers of affected pigs are characterized as histiocytic infiltration and severe diffuse hepatocytic necrosis (3). Renal lesions are mild pyelitis and interstitial nephritis characterized by focal lymphohistiocytic infiltrates with corticomedullary congestion and hemorrhage associated with perivascularitis (39). Many times basophilic cytoplasmic inclusion bodies are observed in various tissues of affected pigs, most commonly in lymph nodes, tonsil, and Peyer's patches of the ileum (81). At present, observation of characteristic histopathological lesions in conjunction with clinical signs is the basis for diagnosing PMWS (33).

Postulated etiology

Type 2 PCV has been most frequently isolated from or detected in pigs with clinical signs and histopathological lesions typical of PMWS (6;22;31;33). The presence of PCV2 has been demonstrated in tissues with histopathological lesions using immunohistochemistry. However, results of animal inoculation studies are inconsistent. The majority of animal trials failed to reproduce any pathological changes or clinical signs in inoculated pigs, although the virus or viral antigens were detected in trachea, lung, liver, kidney, pancreas, lymph nodes, spleen, thymus, ileum, colon, cecum, salivary gland, heart, brain, testis (39;81). On the other hand, in a few studies, inoculated pigs showed mild microscopic changes, such as subtle lymphoid depletion and hepatic necrosis, and some nonspecific clinical signs. (3;39;42). Therefore, Koch's postulates have not yet been fulfilled.

Since pigs inoculated with PCV2 alone did not develop any clinical signs or gross lesions, it has been speculated that other factors may be required in the reproduction of the PMWS-specific clinical signs and lesions (3). Some histopathological lesions characteristic of PMWS such as lymphoid depletion have been reproduced in gnotobiotic or colostrum-deprived pigs concurrently infected with PCV2 and porcine parvovirus (3;23;39;42). Diagnostic observations suggested that it is common that animals affected by PMWS are concurrently infected with PRRSV and PCV2 (2;90). Such animals showed more severe clinical manifestations than animals infected with PRRSV alone. In one recent publication, lesions and clinical signs of PMWS were reproduced in colostrum-fed pigs by PCV2

inoculation followed by injection of commercial *Mycoplasma hyopneumonia* and *Actinobacillus pleuropneumoniae* vaccines. Approximately 21% of treated pigs developed clinical signs typical of PMWS and histopathological changes. The percentage of animals that were diagnosed with PMWS in this particular study was similar to that of morbid animals in a farm affected by PMWS. In addition, management and environmental factors are also considered to contribute to the development of PMWS.

Control and prevention

From the time of the first report of PMWS in the swine population, there has been no good method for controlling this syndrome and preventing the virus spreading among animals. No vaccine is commercially available at present. Porcine circovirus is known to be very hard to inactivate and strongly resistant to environmental stress (34;103). No therapeutic treatments that can be used in the field have been reported. Consequently, it appears that good management practices help reduce the risk for PMWS. For example, all-in/all-out production systems, feeding pigs with multivitamins, minimizing stress, and handling pigs with extra care may have some benefit. However, high rates of mortality were still observed in some cases even after implementing good management practice (8). Segregated early weaning (SEW) has also been practiced with some preventive effect on this syndrome since most of the pigs are known to be weaned negative and then exposed to PCV during the growing period (100). In SEW, pigs are

weaned and removed to a remote area while maternal antibodies are still at protective level.

Administration of corticosteroid appears to be beneficial for reducing the severity of disease and death loss during an acute outbreak of PMWS. Treatment with 0.5 mg/kg corticosteroid intramuscularly improved pig condition after 48 hrs, and decreased mortality dramatically (8). This observation suggests that the syndrome may be an the immune-mediated disease since corticosteroid has effects on the immune system.

Statement of the problem

Porcine circovirus or PCV1 was first identified in 1974 (106) as a noncytopathic viral contaminant of PK-15 cell line and has been known to be nonpathogenic to swine except that the virus has been implicated in congenital tremor in newborn piglets. In the late the 1990's, PCV2, which is genetically and antigenically distinct from PCV1, was recognized in pigs affected by postweaning multisystemic wasting syndrome (PMWS) which is characterized clinically by progressive weight loss and pathologically severe lymphoid depletion. While PCV2 has been reportedly associated with PMWS during diagnostic investigation, Koch's postulates have not been fulfilled yet through experimental animal trials with PCV2. In addition, PCV2 appears to be prevalent in the swine population; yet it is in question why such a small proportion of animals in a given herd are clinically affected by PMWS if PCV2 is responsible for the syndrome. Consequently, the role

of PCV2 in PMWS needs to be assessed and the pathogenesis of PCV2 infection studied.

CHARACTERIZATION OF IMMUNE RESPONSE OF YOUNG PIGS TO PORCINE CIRCOVIRUS TYPE 2 INFECTION

A paper published in the Journal of Viral Immunology

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ABSTRACT

A longitudinal study was conducted to characterize the immune response of young swine to infection with porcine circovirus type 2 (PCV-2). Five 8-week-old caesarian-derived, colostrum-deprived pigs were inoculated intranasally and intramuscularly with a field isolate of PCV-2 at a concentration of 10^4 TCID₅₀/ml. Along with monitoring for clinical signs and viremia, serum samples were collected from all pigs at day 0 and thereafter every 7 days post inoculation (PI) until the termination of the study on day 35 PI. No clinical signs were observed in any of the animals during the study period. In all pigs, PCV-2 was detected by PCR in serum samples collected on days 7, 14, and 21 PI. Viral DNA and antigens were detected by *in situ* hybridization and immunohistochemistry in tonsil, spleen, medial iliac lymph nodes, and ileum collected from each pig at the end of the study.

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Collectively, naïve young swine were shown to be susceptible to PCV-2. Virus-specific antibody was detected by an indirect fluorescent antibody (IFA) assay on day 14 PI, but virus neutralizing antibody was not detected until day 28 PI. As neutralizing antibodies developed, cross-reactivity with PCV type 1 (PCV-1) also developed on the IFA test. Western immunoblot analysis revealed 3 PCV-2 proteins with molecular masses of 28kD, 28.5kD, and 35kD. The 35kD protein was also demonstrated in PCV-1, suggesting that this protein induced the cross-reactivity between PCV types 1 and 2. Antibody to the 28kD protein was detected on day 14 PI and later, indicating that this protein was the most immunogenic. Because of its immunogenicity and specificity to PCV-2, the 28kD protein might provide the antigenic basis for the development of diagnostic tests for detection of PCV-2 antibody.

INTRODUCTION

Porcine circovirus (PCV) is a small, negative-sense, single-stranded DNA virus (19, 27) and was first detected as a noncytopathic viral contaminant of a continuous pig kidney (PK-15) cell line (5, 29). Morphologically, the virus is a nonenveloped virion with icosahedral symmetry (20, 27). The size of the virus is 15-17 nm in diameter. The genome is 1.76 kilobases in size. Along with chicken anemia virus, psittacine beak and feather disease virus, and pigeon circovirus, PCV is classified as a member of Family *Circoviridae* (18). No sequence homology and antigenic relatedness have been demonstrated among these circoviruses (30).

Several independent serologic surveys of swine in Canada, Germany, United States, and other countries revealed that the virus is highly prevalent in domestic swine (5, 6, 12, 26, 28). Antibodies cross-reactive to PCV were also detected in humans, mice, and cattle (25). Despite the common occurrence of PCV antibodies in swine, the virus has not been associated with a specific disease in pigs (1, 28). No clinical disease or pathological changes have been demonstrated in pigs experimentally infected with PCV (1).

Recently, a disease termed "postweaning multisystemic wasting syndrome" (PMWS) was reported in swine. In 1996, the syndrome was described in "high health" status swine herds in Canada and, shortly thereafter, in the United States and Europe (2, 4, 7, 13, 17, 22, 23). The cardinal clinical manifestation of PMWS is progressive weight loss in pigs at 4- to 16-weeks of age (2, 11, 17, 23). Other commonly reported clinical signs include tachypnea, dyspnea, icterus, and diarrhea. Characteristic histopathological changes observed in affected pigs include a varying degree of depletion/atrophy of lymphoid tissues and granulomatous inflammation in any tissue, particularly lymphoid tissues, lung or intestine (8, 10, 14, 23).

A circovirus that is genetically and antigenically distinct from the PCV originally found in a PK-15 cell line has been repeatedly isolated from and/or detected in pigs diagnosed with PMWS (2, 3, 9, 14, 16, 21). The overall DNA sequence homology between these two viruses was reported to be 68-76% (9, 20, 21). To reflect such a difference, it has been proposed to designate the field virus PCV type 2 (PCV-2) and the PK-15 cell contaminant PCV type 1 (PCV-1) (20). A computer-aided analysis of PCV-2 genomic sequences has suggested that the virus may contain as

many as 11 open reading frames (ORFs) (9). The predicted size of proteins encoded by individual ORFs is reported to be approximately 36, 28, 12, 7, 6, 3, 2, 2, 5, 4, and 2 kilodaltons (kD), respectively. ORFs 1 and 2 are considered to encode for two major structural proteins of PCV-2. Nucleotide sequence identity of ORFs 1 and 2 between PCV-1 and PCV-2 was determined to be 83-85% and 66-67%, respectively (9, 21).

However, actual protein constituents of PCV-2 and their immunobiological roles have not been characterized. Furthermore, at the present time, there are no published reports documenting clinical disease and pathological changes in pigs experimentally infected solely with PCV-2, although one study revealed that pigs developed antibody to the virus after infection (8). The following animal trial was conducted to characterize immune responses of pigs at an age apparently susceptible to infection with PCV-2. In the course of the study, clinical and pathological responses of pigs to PCV-2 infection were also assessed.

MATERIALS AND METHODS

Experimental design. A longitudinal study was conducted. Five, 5-week-old cesarean-derived, colostrum-deprived pigs were obtained and housed in the biosafety level 3 animal facility at the National Animal Disease Center, USDA/ARS, Ames, Iowa, USA. Prior to inoculation, pigs were tested twice for the presence of serum antibodies against PCV types 1 and 2 by an indirect fluorescent antibody (IFA) test at 2-week intervals and confirmed negative for both types of PCV. The pigs were also tested for serum antibodies against major swine pathogens, i.e.,

porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease virus (ADV), transmissible gastroenteritis virus (TGEV), swine influenza virus (SIV), porcine parvovirus (PPV), hemagglutinating encephalomyelitis virus (HEV), porcine enteroviruses (PEV), and porcine cytomegalovirus (PCMV). At approximately 8 weeks of age each animal was inoculated with an isolate of PCV-2 recovered from a pig diagnosed with PMWS. Each animal was exposed via intranasal (1 ml/nosril) and intramuscular (2 ml/pig) routes with suspension containing virus at 10^4 TCID₅₀/ml. All pigs were monitored for 5 weeks post inoculation (PI) and clinical signs, such as nasal discharge, skin color, skin lesions, locomotion, appetite, and attitude, were recorded daily. A single investigator made all clinical observations. Pigs were bled on the day of inoculation and every 7 days thereafter during the study period. Serum was separated and stored at -80°C until tested. Viremia was monitored by a polymerase chain reaction (PCR) assay. Antibody response to PCV-2 and PCV-1 was assessed using an indirect fluorescent antibody (IFA) test, a virus neutralization (VN) assay, and western immunoblotting. At the end of the study, all animals were euthanized and tissues collected in 10% neutral buffered formalin for histopathological examination (lung, tonsil, spleen, liver, kidney, ileum, medial iliac lymph nodes). Formalin-fixed tissues were also assayed for the presence of PCV-2 by *in situ* hybridization (ISH) and immunohistochemistry (IHC) techniques. Serum samples collected at the end of the study were checked for antibodies against the swine pathogens described above and classical swine fever virus (CSFV) at the National Veterinary Service Laboratories (NVSL), USDA/APHIS/VS, Ames, Iowa, USA. In addition, the samples were tested by IFA

and VN tests against 4 other field isolates of PCV-2 obtained from cases that were submitted to the Iowa State Veterinary Diagnostic Laboratory (ISUVDL) and diagnosed as PMWS.

Virus and cell. A field isolate of PCV-2 designated ISUVDL 98-15237 was used to challenge pigs in the study. The virus was isolated using PK-15 cells at the ISUVDL from tissues collected from a pig originating from a herd undergoing a clinical outbreak diagnosed as PMWS. The virus was purified by ultra filtration using a series of filters (Fisher Scientific, Pittsburgh, PA, USA), the final filter pore size being 20 nm in diameter. The absence of PCV-1 in the virus preparation was confirmed by both ISH and PCR prior to use. The virus used in this study represented six *in-vitro* passages in cell culture. The complete virus sequence can be found in GenBank (accession number AF147751).

Polymerase chain reaction. A multiplex PCR assay was developed and used to detect and differentiate between the nucleic acid of PCV types 1 and 2 in serum. DNA was extracted from 0.2 ml of each serum sample using QIAamp Tissue Kit (Qiagen, Santa Clarita, CA, USA), as recommended by the manufacturer. Two µl of each extract were used for amplification. PK-15 cells infected with PCV-2 and day 0 serum samples were used as positive and negative controls, respectively. Primers were designed based on nucleotide sequences of type 1 and 2 porcine circoviruses available from GenBank. Primer 1C478 (5'-CCGCGGGCTGGCTGAACTT-3') was designed to amplify both PCV-1 and PCV-2. Primers 1C1108 (5'-CTCGGCTATGCGCTCCAAAATG-3') and 2C16R(5'-ACCCCGCCACCGCTACC-3') were designed to detect PCV-1 and PCV-2,

respectively. The PCR was performed in 35 cycles with the following parameters: denaturation at 94°C for 20 seconds, annealing at 62°C for 20 seconds, and elongation at 72°C for 20 seconds. Amplification products were separated on a 1.5% agarose gel and stained with ethylene bromide. Samples containing PCV-1 and PCV-2 resulted in 1154-bp and 652-bp products, respectively.

Indirect fluorescent antibody test. The IFA test was performed to detect antibody specific for PCV-1 and PCV-2, as previously described (2, 5). A suspension of PK-15 cells was prepared in MEM (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and an antibiotics-antimycotic mixture (MEM growth medium) at a concentration of 4×10^5 cells/ml. Type 2 PCV antigens were prepared by adding the virus ($10^{4.5}$ TCID₅₀/ml) to the suspension of PK-15 cells at a ratio of 1:50, seeding inoculated cells in 96-well plates, and incubating the cells at 37°C in a 5% CO₂ atmosphere. After 20 to 24 hours of incubation, the cells were treated (25 µl/well) with 300 mM D-glucosamine (Sigma Chemical Co., St. Louis, MO, USA) prepared in MEM for 20 minutes. Cell monolayers were washed once with MEM supplemented only with the antibiotics-antimycotic mixture and replenished with 200 µl of fresh MEM supplemented with 15% FBS and the antibiotics-antimycotic mixture. The cells were incubated for another 16 hours, then fixed in cold 80% aqueous acetone. Type 1 PCV antigens were prepared in the same manner. Uninfected PK-15 cells served as negative control antigens.

For the IFA test, serum samples were serially diluted 2-fold in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. Each diluted sample was added to

PCV-2 infected or PCV-1 infected cell monolayers, as well as negative control cells. Plates containing the antigen-serum mixture were incubated for one hour at 37°C. The cells were rinsed with PBS three times. The antigen-antibody reaction was visualized by staining the cells with optimally diluted goat anti-porcine IgG (H+L) conjugated with fluorescein isothiocyanate (Kirkegard and Perry Laboratories, Inc., Gaithersburg, MD, USA). Antibody titers for individual samples were determined as the reciprocal of the highest dilutions in which specific fluorescence staining was observed, as illustrated in Figure 1.

Virus neutralization test. The VN test was performed in 96-well microtitration plates using PK-15 cells as the indicator. Serum samples were heat-inactivated at 56°C for 30 minutes prior to performing the test. In a 12 x 8 format, 100 µl of MEM growth medium were added to wells, starting at the second well of each row in a 96-well plate. Each serum sample was then added to the first well that did not contain the medium and serially diluted 2-fold up to 1:512. The first well was used as serum control for each sample to check the cytotoxicity. The last two wells were used as mock-infected and virus-infected cell controls, respectively. One hundred µl of PCV-2 at a rate of 200 TCID₅₀/0.1 ml were added to each well containing an equal volume of each sample dilution except the first and second last wells. Plates containing mixtures of virus-serum or virus-medium, medium only, and serum only were incubated at 37°C for 60 minutes. After that, 100 µl of the cell suspension prepared in MEM growth medium at a concentration of 4×10^5 cells/ml were added to each well.

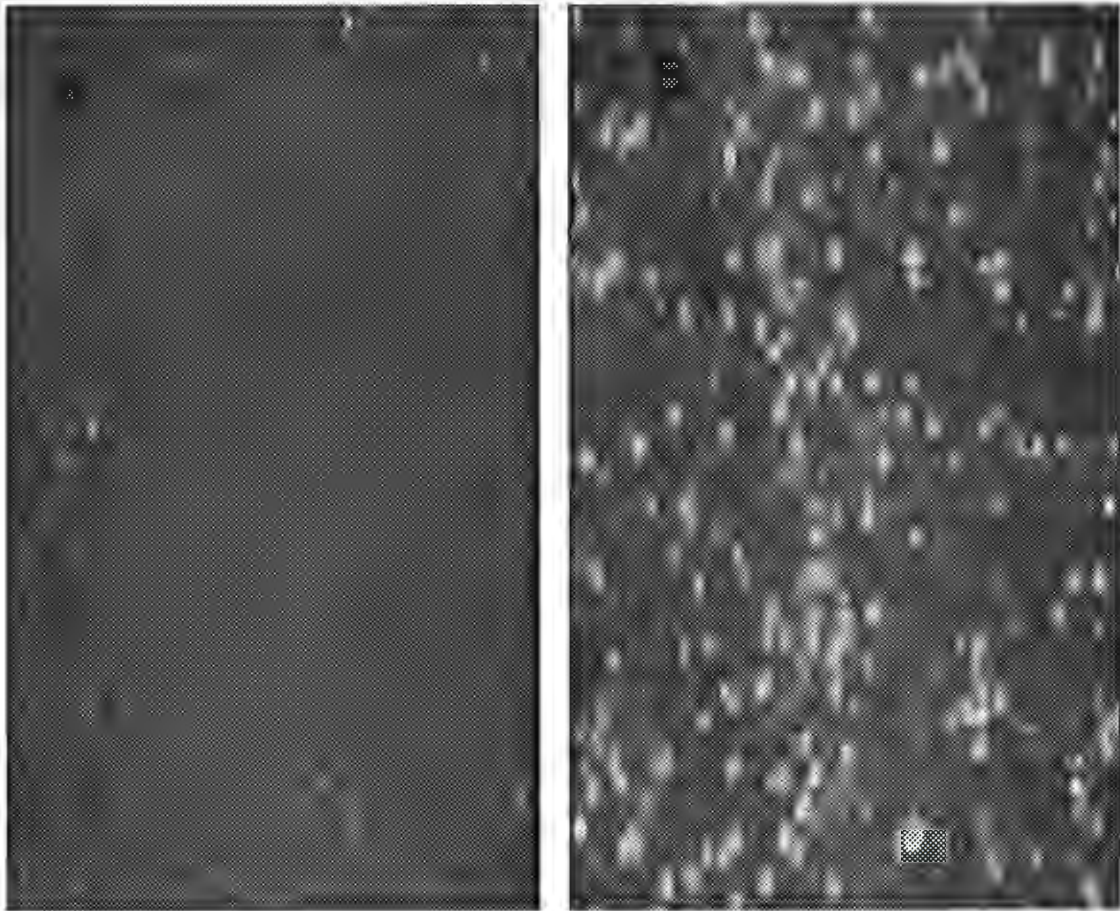


Figure 1. Immunofluorescence microscopy on PK-15 cell monolayers not infected (A) and infected (B) with PCV-2 using PCV-2 specific hyperimmune serum. Infected cells show intensive nuclear staining.

Plates were placed in a 37°C humidified CO₂ incubator. After a 48-hour incubation, the resulting cell monolayers were treated with 300 mM D-glucosamine, as described above. After an additional 16 hours of incubation, the cells were fixed in cold 80% acetone. Since no visible cytopathic effect was expected, growth or suppression of virus replication in inoculated cells was confirmed by immunofluorescence microscopy and the number of fluorescent foci was counted. Virus neutralizing antibody titers were expressed as the highest dilution in which no or higher than 80% reduction of virus replication was detected as compared to the virus control.

Preparation of the antigen for western immunoblot analysis. A PK-15 cell suspension was prepared in MEM growth medium at a concentration of 4×10^5 cells/ml and inoculated with PCV-2 of 10^5 TCID₅₀/ml at a ratio of 1 ml virus per 50 ml cell suspension. Inoculated cells were seeded into a 165-cm² tissue culture flask and incubated for 24 hours. The resulting cell monolayers were treated with 300 mM D-glucosamine for 30 minutes and incubated for another 48 hours. Virus-infected cells were then freeze-thawed twice, harvested using a cell scraper, and pelleted by centrifugation at 3,000 x g for 10 minutes. Supernatant was saved as virus antigens. The cell pellet was then resuspended and disrupted in a lysis buffer (0.05 M Tris, 0.15 M NaCl, 0.002 M EDTA, 0.1% SDS, 1% TritonX-100, 0.1% sodium azide, pH 8.0) at a rate of 1 ml buffer per 0.1 ml of the cell pellet. The suspension was stirred for 15 minutes at 4°C and centrifuged at 3,000 x g for 10 minutes. The supernatant was mixed with the cell culture supernatant that was saved earlier and ultracentrifuged at 190,000 x g for 3 hrs. The pellet was

resuspended and disrupted in the lysis buffer at 4°C for another 6 hours. PCV-1 and negative cell control antigens were prepared in the same manner. The protein concentration in each sample preparation was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) following the manufacturer's recommended procedures and adjusted to 2 mg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A modified Laemmli procedure was used for protein separation (15) on a discontinuous slab gel (70 x 80 x 0.75 mm) consisting of 5% stacking gel and 12.5% resolving gel cross linked with bis-acrylamide (Bio-Rad Laboratories, Richmond, CA, USA). The viral (PCV-1 and PCV-2) and cell antigen preparations described above were solubilized in a sample buffer (pH 6.9) containing 0.0625 M Tris, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol by boiling at 100°C for 5 minutes. Fifteen microliters of each denatured sample (10 µg protein) and 10 µl of prestained SDS-PAGE molecular standards (Bio-Rad Laboratories, Richmond, CA, USA) were loaded on the gel. The molecular weight standard contained 8 proteins with molecular masses of approximately 206, 117, 79, 48.3, 34.7, 29.3, 21.3, and 7.6kD. Electrophoresis was carried out using a vertical mini-gel apparatus, as directed by the manufacture.

Electrophoretic transfer of the proteins. A mini-trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA, USA) was used following the procedures recommended by the manufacture. Viral and cellular proteins, along with the molecular weight markers that were separated in the gels, were electrophoretically transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad

Laboratories, Richmond, CA, USA) following SDS-PAGE. Transfer was carried out at 4°C for 60 minutes in a transfer buffer (pH 8.3) consisting of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Nitrocellulose membranes containing viral and control cellular antigens were blocked overnight at 4°C with 1% bovine serum albumin dissolved in Tris-buffer saline (TBS, pH 7.5) containing 500 mM NaCl and 20 mM Tris.

Western immunoblotting. Blocked membranes were washed twice for 5 minutes each in gently agitating TBS and cut into strips containing viral antigens, cellular antigens, and molecular weight standards. Serum samples to be tested were diluted 1:50 in TBS containing 0.05% Tween 20 (TTBS) and 1% bovine serum albumin. Sera collected from pigs on the day of inoculation served as negative controls. Each diluted serum sample was added to the single membrane strip and incubated for 1 hour at room temperature. Following incubation, the membranes were washed 3 times in TTBS by gently agitating for 5 minutes each. Antigen - antibody reactions were visualized with diluted goat anti-swine IgG (H+L) labeled with horseradish peroxidase and TMB membrane peroxidase substrate (Kirkegard and Perry Laboratories, Inc., Gaithersburg, MD, USA). The color reaction was stopped by brief washes in deionized water. The appearance of virus-specific reactivity was assessed by comparing the antibody responses to the viral and cellular antigens.

Histopathology. Tissues were collected and fixed by immersion in 10% neutral buffered formalin. Fixed tissues were processed, embedded, and sectioned following standard procedures. Each section was then stained with hematoxylin and eosin.

Immunohistochemistry and *in situ* hybridization. Tonsil, liver, ileum, kidney, lung, medial iliac lymph node, thymus, and spleen were collected from each pig, fixed in 10% neutral buffered formalin, and tested for the presence of nucleic acid and antigens of PCV-2 by ISH and IHC, respectively. The ISH was performed using fluorescein-labeled sense and antisense RNA probes specific for ORF1 of PCV-2 (ISU-31), as previously described (21). In brief, tissue sections (5 µm thick) were deparafinized, rehydrated, and treated with proteinase K (50 µg/ml). Before hybridization, sections were incubated with 200 µl of hybridization buffer using a CoverWell® chamber (Grace Bio-Labs, Bend, OR) at 95°C for 10 minutes. Sections were hybridized with the RNA probes, washed, and treated with RNase A. The presence of PCV-2 nucleic acid was visualized by incubating each section with anti-fluorescein alkaline phosphatase and then 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim, Indianapolis, IN). Sections were counter stained with nuclear fast red prior to microscopic examination.

The IHC was performed using hyperimmune rabbit anti-PCV-2 serum raised against highly purified ISU-31 isolate of PCV-2, as described elsewhere (24). In brief, tissue sections (4 µm thick) were deparafinized, rehydrated, and treated with proteinase K. Each section was incubated with optimally diluted PCV-2 antiserum. Antigen-antibody complexes were visualized using a biotin-streptavidin-diaminobenzidine system, DAKO LSAB2® kit (DAKO Corporation, Carpinteria, CA). Sections were counter stained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA).

Statistical analysis. Differences in serologic responses among isolates of PCV-2 and PCV-1 in the IFA and VN tests were evaluated using the analysis of variance and Duncan's multiple range test.

RESULTS

No clinical signs resembling those described for PMWS were observed after experimental inoculation. All inoculated pigs remained normal in their appearance and behavior throughout the study period. As determined by the detection of PCV-2 DNA in serum by PCR, all pigs became viremic after inoculation (Figure 2). Viremia was detected in all pigs on days 7 and 14 PI and in 2 of 5 pigs on day 21 PI. No pigs were viremic on day 28 PI. No PCV-1 was detected by PCR in the serum of any of the 5 pigs during the study period.

The humoral immune response of the pigs to PCV-2 is summarized in Figure 3. Two weeks prior to and on the day of challenge, none of the 5 pigs had detectable antibody against either PCV-2 or PCV-1 as measured by IFA and VN tests. After challenge all pigs seroconverted to PCV-2. Virus-specific IgG antibody was initially detected by IFA at 14 days PI (1:80 to 1:640). Antibody levels continued to increase and by the end of the study the mean antibody titer was 1:1792 with a range of 1:1280 to 1:2560. Day 35 serum samples were also assayed by IFA using 4 other field isolates of PCV-2 in the test system. No significant difference in antibody titers to individual isolates was observed when compared to that against the homologous isolate ($p>0.01$).

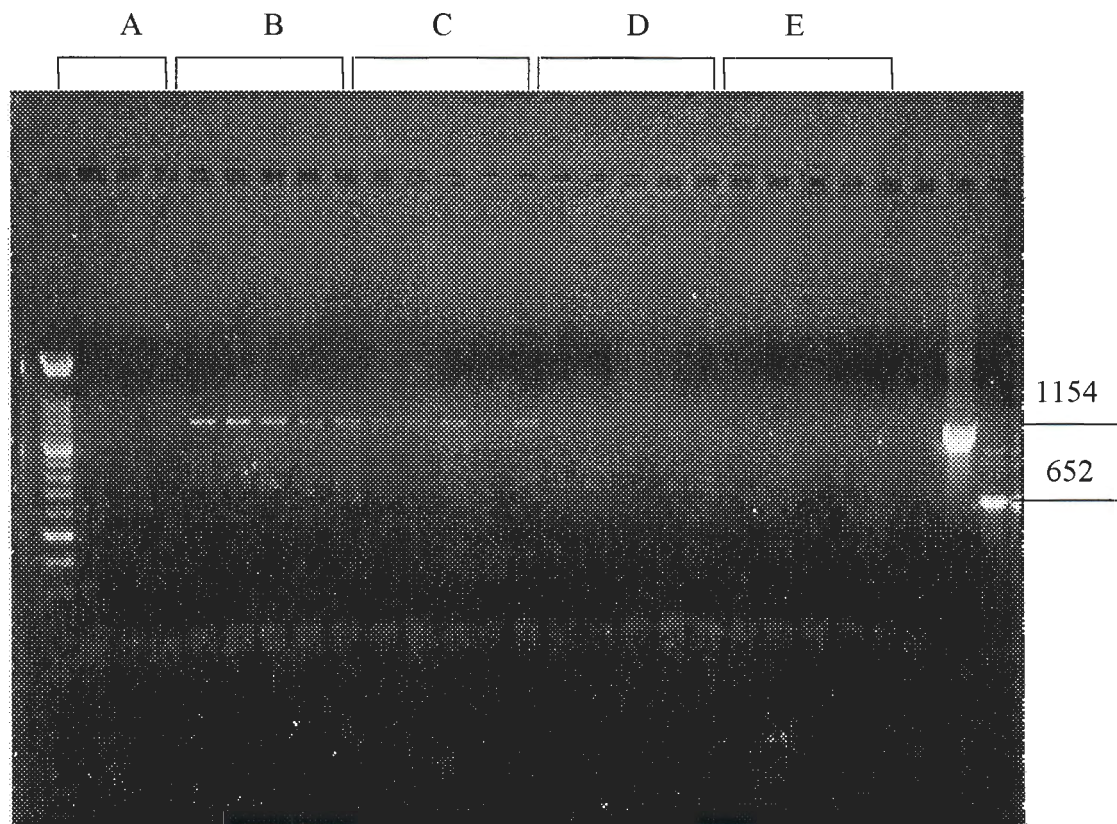


Figure 2. Detection of cell-free viremia of PCV-2 in 5 experimentally infected pigs by a multiplex polymerase chain reaction. Lane 1: 100-bp DNA ladder; A-E: sera collected at day 0, 14, 21, and 28 post inoculation, respectively; Last 3 lanes are negative control, PCV-2 positive control, and PCV-1 positive control, respectively.

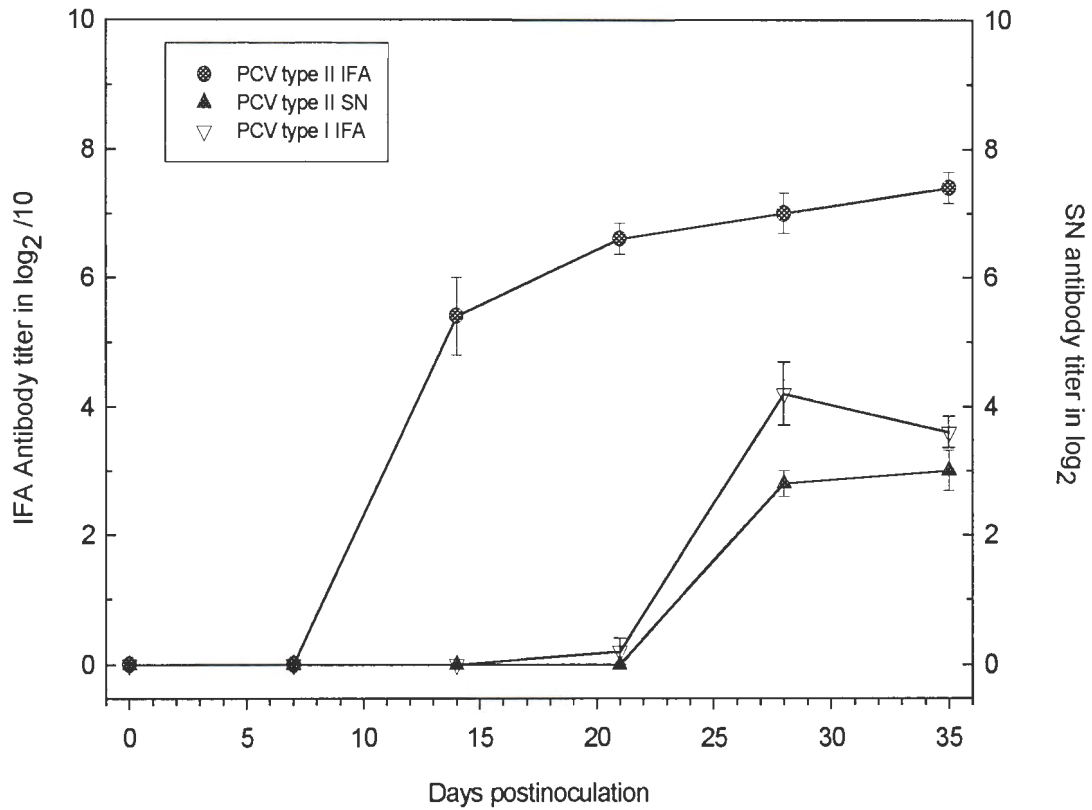


Figure 3. Antibody responses of 5 CD/CD pigs to PCV-2 infection as determined by indirect fluorescent antibody (IFA) test (●) and virus neutralization (VN) test (▲). Serum samples were also tested by IFA test for antibody cross-reactive to PCV-1 (▽). Each point represents mean \pm SEM.

Antibody titers against the 4 field isolates ranged from 1:1280 to 1:2560.

Neutralizing antibody against PCV-2 was first detected on day 28 and on day 35 the mean VN titer was 1:8.8. Using sera collected on day 35, no significant difference in susceptibility to neutralization was observed among the 4 additional PCV-2 isolates. As neutralizing antibody to PCV-2 appeared, cross-reactivity with PCV-1 was also detected by IFA in all pigs. However, IFA antibody titers to PCV-1 ranged from 1:160 to 1:320 and were significantly lower than IFA antibody titers to PCV-2 ($p<0.01$). No virus neutralization against PCV-1 was detected either.

No antibody to other significant swine viral pathogens was detected throughout the study period, including CSFV, HEV, PCMV, PEV, PPV, PRRSV, PRV, SIV, and TGEV.

Under reducing conditions, 3 polypeptides with molecular masses estimated at 28, 28.5, and 35kD were demonstrated in PCV-2 using day 35 serum samples as a source of antibody (Figure 4A). The antibody response indicated that the 35kD protein was associated with PCV-1, as well. The temporal viral protein specificity of the immune response was also characterized by western immunoblot analysis (Figure 4B). Antibodies reactive to the 28kD protein were first detected in pigs at 14 days PI and antibodies to the 28.5kD and 35kD proteins were detected on day 21 PI. Once antibody to each viral polypeptide had appeared, it persisted to the end of the study.

At necropsy (35 days PI), no gross lesions were observed in any of the 5 pigs. Microscopically, livers from all pigs had small foci of individual hepatocyte necrosis associated with aggregates of lymphocytes and plasma cells. In addition, apparent

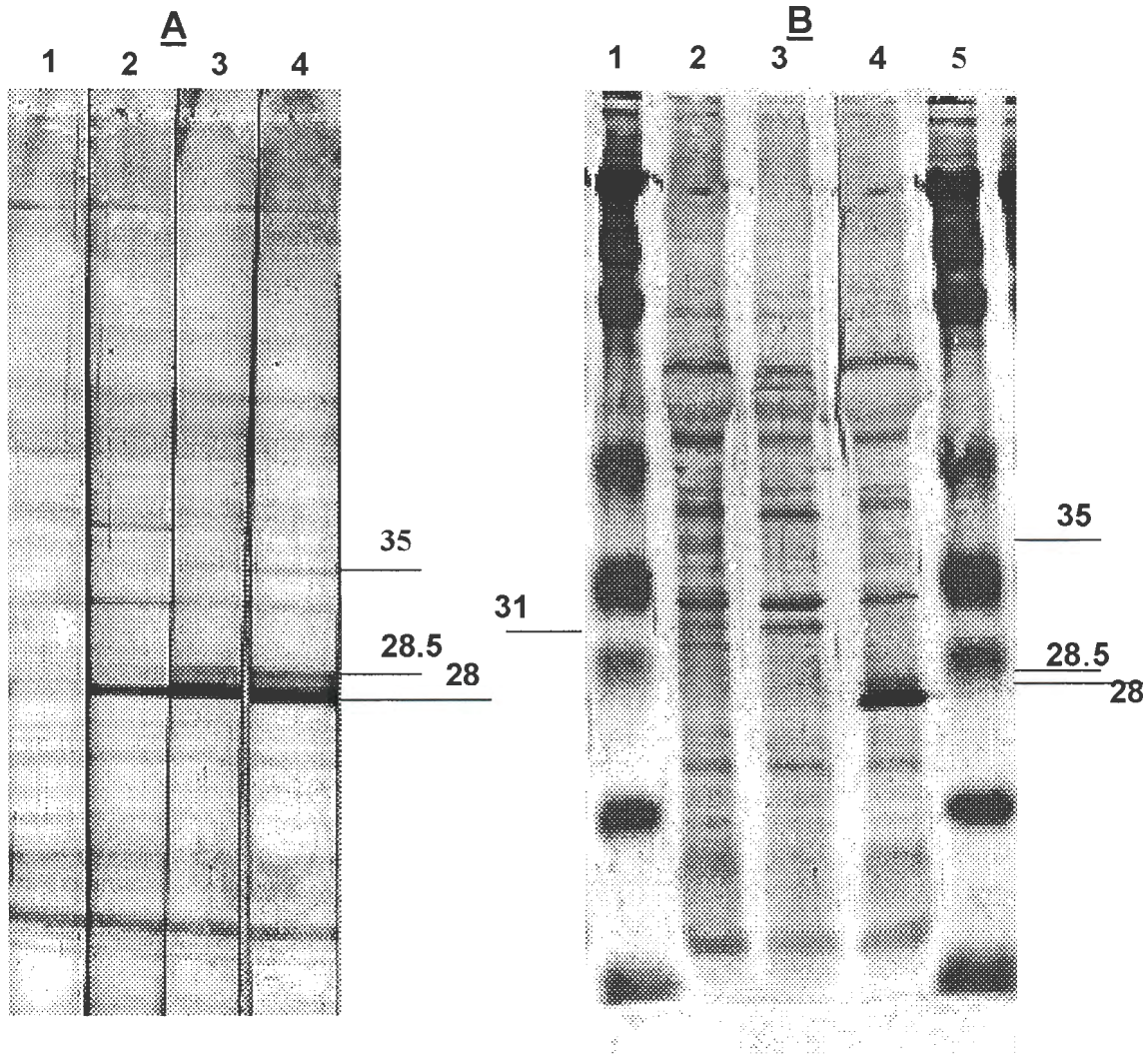


Figure 4. Representative western immunoblot analysis of the antibody response of pigs to PCV. Panel A is a photograph showing the temporal viral protein specificity of antibody responses of 5 pigs to PCV-2 infection using pooled sera from the pigs on days 7 (lane 1), 14 (lane 2), 21 (lane 3) and 28 (lane 4) post inoculation. Panel B illustrates the reactivity of antibodies in pooled sera that were collected from PCV-2 infected pigs on day 28 PI to PCV-1 (lane 2), negative control cell (lane 3), and PCV-2 (lane 4) antigens. Lanes 1 and 5 are protein molecular weight markers.

mild depletion of lymphoid follicles was observed in tonsil, spleen, and Peyer's patch of the ileum. Lungs and other tissues examined were unremarkable. In 4 of the 5 pigs, the viral genome and viral antigens were detected by ISH and IHC, respectively, in the tonsil, Peyer's patches of the ileum, and medial iliac lymph nodes (Figure 5).

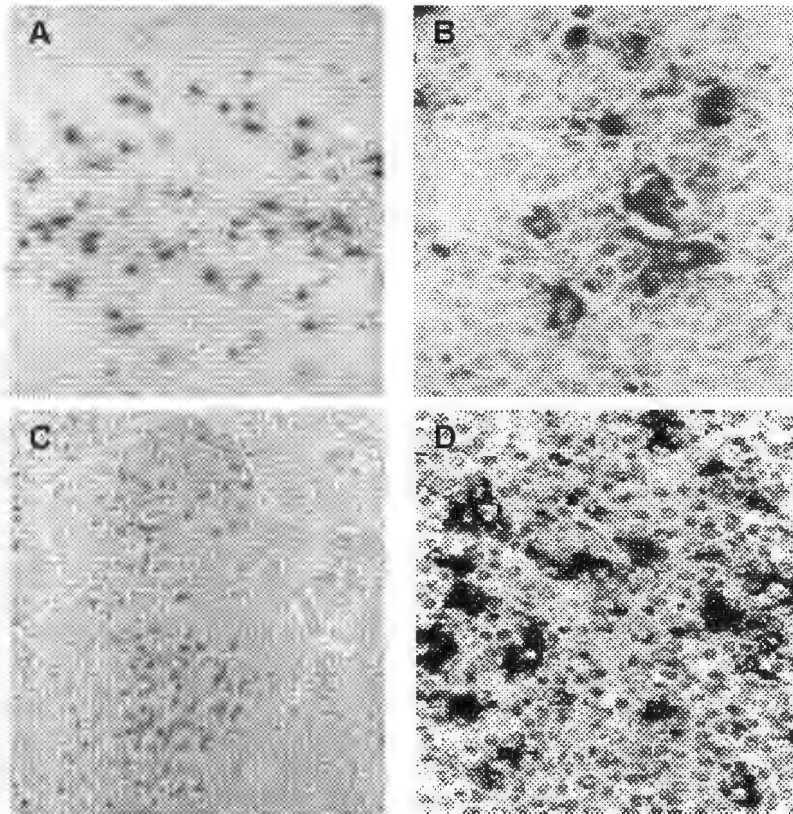


Figure 5. Photomicrographs of tonsil (A, B) and lymph node (C, D) collected on day 35 post inoculation from one of 5 pigs experimentally infected with PCV-2 in which the virus was detected by *in situ* hybridization (A, B) and immunohistochemistry (C, D), respectively.

DISCUSSION

The goal of this study was to assess the immunological response of naive pigs to PCV-2 infection under experimental conditions. After inoculation, PCV-2 viremia was apparent through day 21. At the termination of the study, the virus was still present in various tissues of inoculated pigs in which lymphoid depletion, the characteristic microscopic lesion of PMWS, was observed, although the degree of depletion was very mild. In addition, mild hepatic lesions, similar to those previously reported to be associated with PCV-2 infection, were also observed (8, 11). These results clearly indicated that naive pigs were susceptible to PCV-2. However, lack of clinical signs and remarkable pathologic changes compatible with PMWS suggest that, if PCV-2 has a causal role in PMWS, the presence of additional factors may be necessary to induce PMWS. This point remains to be clarified.

Although questions remain as to the pathogenesis of PMWS, this study contributed to our basic understanding of virus structure and host response. The protein composition of PCV-2 is unknown, but based on an analysis of the viral genome, PCV-2 has been postulated to contain as many as 11 ORFs each of which could encode for a different polypeptide (9). However, western immunoblot analysis in this study demonstrated only 3 viral polypeptides with molecular masses of 28, 28.5, and 35kD (Figure 4). Of these, the 28 and 35kD proteins matched the expected molecular weight of the polypeptides predicted to be encoded by ORFs 1 and 2 (9, 21) and, on that basis, may be postulated to be the protein products of ORF 2 and 1, respectively. Because of the reducing conditions required to prepare samples for western immunoblotting, it is possible that viral proteins present in the

PCV-2 preparation underwent conformational changes and were no longer recognized by antibodies.

The 28kD protein was the most immunogenic of the 3 proteins identified, based on the observation that antibodies specific for this protein appeared first and persisted to the end of the study (Figure 4B). Assuming that the intensity of the reaction to a protein in western immunoblotting reflects the quantity of antibody in serum, levels of antibodies against the 28kD protein in sera at a given dilution were always much higher than antibodies to the other two proteins. Furthermore, antibody against the 28kD protein was not detected using PCV-1 antigens on western immunoblotting. These results suggested that the 28kD protein might provide the antigenic basis for the development of diagnostic tests for the detection of PCV-2-specific antibody.

Results of western immunoblot analysis and serological monitoring data suggested that the 28.5kD and/or 35kD proteins were associated with the induction of neutralizing antibody and the 28kD protein was not. Antibody to the 28kD protein was present in all pigs for 2 weeks prior to the initial detection of neutralizing antibody. It is not certain whether the 28.5kD protein, the 35kD protein, or both are responsible for virus neutralization, but neutralizing activity in sera appeared to correlate with the appearance of antibodies to the 35kD protein. As shown in Figure 4B, the antibody response to the 35kD protein was very weak on day 21 PI and became stronger at day 28 PI when detectable VN antibody was present. In contrast, an intensive reaction was already detected with 28.5kD protein at day 21 PI in the absence of detectable neutralizing antibody. Additional research is

necessary to define the role of antibodies specific for 28.5kD and 35kD protein in viral neutralization.

Porcine circovirus type 2 has been reported to be genetically and antigenically distinct from PCV-1 (2, 9, 21). In this study, marked antigenic differences between PCV-1 and PCV-2 were demonstrated on the basis of significant differences in IFA antibody titers and antibodies specific for PCV-2 failed to neutralize PCV-1 (Figure 3). In addition, western immunoblot analysis demonstrated 3 viral proteins that have not been reported in association with PCV-1. Previously, two immunoreactive proteins with a molecular mass of 31kD and 33kD were reported for PCV-1 (26). These proteins however were not detected in this study, either because it was not present in PCV-2, or if present, it may have been subjected to conformational changes brought about by the reducing conditions necessary for the preparation of viral antigens for western immunoblotting.

Cross reactivity between PCV-1 and PCV-2 in IFA test has been reported (2). Although the data strongly supported significant differences between PCV-1 and PCV-2, some degree of serologic cross-reactivity between PCV-1 and PCV-2 was demonstrated in the IFA test (Figure 3). At the protein level, antibodies to the 35kD protein appeared responsible for the cross-reaction. This conclusion is based on the fact that antibody from pigs infected with PCV-2 recognized this protein in both types of PCV on western immunoblotting (Figure 4). Assuming that the 35kD protein is the protein product of ORF1, our conclusion is also supported by sequence analysis data demonstrating high DNA homology in ORF1 between PCV-1 and PCV-2 (9, 20, 21). Previous serological surveys using PK-15 contaminated

with PCV, i.e., PCV-1, demonstrated that PCV infection was common in swine. Data from the present study showed that earlier serological surveys would have detected antibodies against either PCV-1 or PCV-2. That being the case, PCV-2 may have been present in the swine population for quite some time and not a recent introduction.

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CASE-CONTROL STUDY ON THE ROLE OF PORCINE CIRCOVIRUS TYPE 2 AND OTHER MAJOR SWINE VIRUSES IN POSTWEANING MULTISYSTEMIC WASTING SYNDROME

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Abstract

A field-based case-control study was conducted to assess the strength of association of porcine circovirus type 2 (PCV2) and some major swine viruses with postweaning multisystemic wasting syndrome (PMWS). Cases were defined as individual pigs with a clinical history of progressive weight loss and respiratory signs and that were subsequently diagnosed with PMWS on the basis of histopathological observations: 1) depletion/atrophy of lymphoid tissue and/or 2) macrophage-dominated granulomatous inflammation mainly in lymphoid tissues, lung, and intestine. Controls were pigs without histopathological lesions characteristic of PMWS and/or from herds in which PMWS had not been diagnosed and with no clinical signs compatible with PMWS.

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A total of 31 cases and 56 controls were identified from diagnostic submissions or farms within a six-month period. Serum and various tissues were collected from all animals and assayed for PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus, porcine enterovirus (types 2 and 3), swine influenza viruses, porcine respiratory coronavirus, transmissible gastroenteritis virus, porcine endogenous retrovirus, porcine lymphotropic herpesvirus type 1, and bovine viral diarrhea virus. The proportion of case and controls pigs positive for each virus was determined and statistically compared to determine the strength of the association that each virus had with PMWS. In addition, PCV2 isolates from 6 cases and 4 controls were selected and genetically compared. Type 2 PCV appeared to be strongly associated with PMWS (odd ratio = 9.3) as compared to other viral agents tested for. Risk for PMWS was much higher if the animal was concurrently infected with PCV2 and PRRSV (odd ratio = 31.2). However, PCV2 was also found in many controls (35/56) and was not detected in 2 of the 31 PMWS pigs. Furthermore, no significant genetic difference was observed between PCV2 isolates from PMWS and control pigs, indicating that the difference in virulence, if any, may not be associated with viral genetic trait. Therefore, causal role of PCV2 in PMWS is in question. Development of PMWS may require co-factor(s) in addition to PCV2. Further work remains to determine the actual role of PCV2 in PMWS and the pathogenesis of PCV2 infection.

INTRODUCTION

Postweaning multisystemic wasting syndrome (PMWS) is an emerging problem of growing pigs worldwide (10;17). The syndrome is characterized by progressive weight loss in pigs at 4-16 weeks of age. Other common clinical symptoms are respiratory signs, such as tachypnea and dyspnea, icterus, and diarrhea (17). Pathological changes have been observed in lymphoid organs and lungs of affected pigs. A consistent histopathological finding is the depletion of lymphocytes and replacement with macrophages in the B- and T-cell-dependent areas of all lymphoid tissues. Other common microscopic changes include lymphocytic to granulomatous interstitial pneumonia, lymphadenopathy, and less frequently lymphocytic granulomatous hepatitis and nephritis (6;10;14;36). The PMWS was first recognized in Canada in 1991 as a chronic, insidious and sometimes protracted disease (10;17). Although the etiology of the syndrome is not completely elucidated to date, isolation or detection of circovirus which is genetically and antigenically distinct from PCV originally found in PK-15 cell line as noncytopathic viral contaminant, is reportedly associated with PMWS (6;14). To reflect the significant genetic and antigenic difference between these two circoviruses, the circovirus isolated from pigs affected by PMWS has been designated PCV type 2 (PCV2) (2;30). Genome and/or antigens of PCV2 have been detected within and around lesions in tissues, such as tonsil, multiple lymph nodes, spleen, Peyer's patches, bone marrow, small intestine, kidney, and lung, from pigs affected by PMWS (36).

Although PCV2 is postulated to be the causative agent for PMWS, there are several reports suggesting that PCV2 may not be the only agent causing PMWS. PCV infection in swine herds with and without clinical disease compatible to PMWS has been documented through sero-epidemiological studies, suggesting that PCV is ubiquitous. (5;12;25;43) Despite of its apparent ubiquity, the incidence of PMWS in a given herd is considerably low, 5-15% (38). There are no published reports documenting clinical disease and pathological changes in conventionally raised pigs which were experimentally infected with PCV2 only, although the virus is demonstrated to be infectious for naïve swine based on virus isolation/detection in various tissues and seroconversion (3;15;23;34). In addition, pathological changes compatible with lesions described in field cases have been experimentally reproduced only in pigs concurrently infected with PCV2 and porcine parvovirus (3;7;23;29). It has been speculated that there is the involvement of immunosuppressive agents, management problems, or environmental factors in the development of PMWS (14). Therefore, the causal role of PCV2 in PMWS has not been conclusively demonstrated.

The following case-control study was conducted to evaluate the relative importance of PCV2 and some major swine viral agents in PMWS and determine if genetic trait of PCV2 is associated with its pathogenicity. In part, diagnostic performance of different laboratory tests in detecting PCV2 and its value for PMWS diagnosis was also evaluated.

MATERIALS AND METHODS

Experimental design

The investigation was a field-based case-control study. Cases were pigs at 4 to 16 weeks of age with clinical signs of PMWS (i.e., progressive weight loss, respiratory disease) on farm and later diagnosed as PMWS based on the presence of characteristic histopathological lesions: 1) some degree of depletion/atrophy of lymphoid tissue and/or 2) macrophage-dominated granulomatous inflammation in any tissue, but especially lymphoid tissue, lung, and intestine. Controls were pigs at 4 to 16 weeks of age without PMWS and/or from herds in which PMWS had not been diagnosed and no clinical manifestations described for PMWS were present, or sick pigs submitted with completely unrelated clinical history to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for diagnostic evaluation. A total of 31 cases and 56 controls were identified within a six-month period and used for the study. Serum and various postmortem tissues (tonsil, spleen, lymph nodes, lung, liver, and ileum) were collected from each pig and stored frozen at -80°C until tested. Clinical specimens were assayed by virus isolation (VI), immunohistochemistry (IHC), and/or polymerase chain reaction (PCR) for PCV2 and other viral agents such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), porcine enterovirus types 2 and 3 (PEV), swine influenza virus (SIV), porcine respiratory coronavirus (PRCV), transmissible gastroenteritis virus (TGEV), porcine endogenous retrovirus (PERV), porcine lymphotropic herpesvirus type 1 (PLHV-1), and bovine viral diarrhea virus (BVDV). Appropriate serologic assays were performed to determine the presence and

absence of antibody against some of these viruses. The proportion of pigs virologically and/or serologically positive for each virus was determined for case and control groups and statistically compared to determine the strength of the association that each viral agent had with PMWS individually or in combination. In addition, PCV2 isolates from 6 cases and 4 controls were selected. The entire genome of each isolate was sequenced and compared each other to identify genetic marker(s) unique to PCV2 from PMWS cases. The agreement of various laboratory diagnostic tests in detecting PCV2 was also evaluated, as well as diagnostic performance (i.e., sensitivity, specificity) of each test.

Virus isolation (VI)

Virus isolations were attempted on clinical specimens for PCV2 and PRRSV as described elsewhere (6;22;34). A continuous porcine kidney cell line PK-15 free of PCV1 and MARC145 clone of African Green Monkey kidney cell line (22;45) were used for virus isolation as indicator system for PCV2 and PRRSV, respectively. For better recovery of PCV2 from clinical specimens, PK-15 cells were treated with D-glucosamine as previously described (42). Samples were considered negative for these two viruses after two blind passages. At the end of each passage, the absence of virus in cells was confirmed by immunofluorescence microscopy using appropriate polyclonal or monoclonal antibody specific for each virus.

Polymerase chain reaction (PCR) assays

Various PCR-based assays were used to detect nucleic acids of PCV2 and other viruses in specimens. A multiplex PCR was used for specific detection of PCV2 DNA as previously described (34). Reverse transcription (RT)-PCR assays were used for detecting RNA of PRRSV (46), BVDV (35), PERV (1) and PEV (47). Swine influenza virus or TGEV/PRCV genome in samples was detected using multiplex RT-PCR assays established in ISU-VDL (19); PCR-based assays described by (31) and (13;15) were employed for detecting DNA of PPV and PLHV-1, respectively. In brief, viral DNA or RNA was extracted from 0.2ml of each sample using QIAamp® Tissue Kit (Qiagen, Santa Clarita, CA) as recommended by the manufacturer. Two µl of each extract was used for amplification. In each assay, virus or virus infected cells and mock-infected cells were included as positive and negative controls, respectively. The presence of target sequence in samples was visualized by an agarose (1.5%, FMC NuSieve 3:1) gel electrophoresis of PCR amplicon with 100-bp DNA ladders (GIBCO/BRL, Grand Island, NY, USA) and molecular size determination. PCR primers for each viral agent and detailed thermocycling conditions can be found in references cited previously.

Histopathology and Immunohistochemistry (IHC)

Tissues (tonsil, liver, ileum, kidney, medial iliac lymph node, lung) were fixed by immersion in 10% neutral buffered formalin immediately after collected. Fixed tissues were processed, embedded, and sectioned following procedures established in ISU-VDL. Each section was then stained with hematoxylin and

eosin. The IHC for detecting PCV in tissues was performed using hyperimmune rabbit anti-PCV-2 serum raised against a PCV-2 isolate as described elsewhere (39). In brief, tissue sections (4 µm thick) were deparafinized, rehydrated, and treated with proteinase K. Each section was incubated with optimally diluted PCV-2 antiserum. Antigen-antibody complexes were visualized using a biotin-streptavidin-diaminobenzidine system, DAKO LSAB2[®] kit (DAKO Corporation, Carpinteria, CA). Sections were counter stained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA).

Serology

Various serological assays were performed to determine the presence and titer of antibodies to PCV, PPV, PRRSV, SIV, TGEV, and BVDV. Antibody specific for PCV was assessed using an indirect fluorescent antibody (IFA) test as previously described (34). PRRSV antibodies were measured by a commercial ELISA kit using the procedure recommended by the manufacturer.

Hemagglutination inhibition (HI) test was employed to assess antibodies against PPV and SIV (both H1N1 and H3N2 subtypes). Serum virus neutralization (SVN) tests were used to detect antibodies to BVDV (types 1 and 2) and TGEV/PRCV. Hemagglutination inhibition and SVN tests were conducted using protocols established in ISU-VDL.

Sequence analysis of PCV2

Total DNA was extracted from a homogenate of mixed tissues (tonsil, spleen, lymph nodes, lung, liver, and small intestine) from each of selected animals using DNA QIAamp® Tissue Kit (Qiagen Inc. Valenica, CA, USA) according to protocol recommended by the manufacturer. The genomic DNA of PCV2 was amplified by PCR using 2 sets of overlapping primers that were designed using the sequence data in GenBank (AF147751). PCR products were purified using a PCR purification kit QIAquick® (Qiagen Inc. Valenica, CA, USA) according to manufacturer's protocol. Two overlapping products from two separate PCR reactions were sequenced using a automated sequencer model ABI377™ (Applied Biosystems, Forest City, CA, USA) at the ISU Nucleic Acid Facility. Sequence data were then proofread, aligned and analyzed using computer software, DNASTar™ (DNASTAR, Inc. Medison, WI, USA). PCR and sequencing primers are summarized in Table 1.

Statistical analysis

The distribution of PCV or other virus positives among cases and controls was evaluated statistically using Chi square test. Spearman's rank correlation between agents was assessed to determine if there was any potential multicollinearity type problem if particular agents were highly correlated with each other, i.e., one agent was always present with another (11). Association of more than one agent with PMWS was determined by logistic regression analysis (9). If

Table 1. PCR and sequencing primers for sequencing the whole genome of porcine circovirus type 2

Use	Designation	Nucleotide sequence (5' – 3')
PCR primers:	C931F	AAGGGGGCCAGTTCGTCACC
	C268R	AGCGGGCACCCTAAATACCACTTCA
	C22F	CAGCACCTCGGCAGCACCTCAG
	C1718R	TACAATCCCCCAACCCTTCTCCTG
Sequencing primers:	C22	CAGCACCTCGGCAGCACCTCAG
	C471	CAGCACCTCGGCAGCACCTCAG
	C931	AAGGGGGCCAGTTCGTCACC
	C1718	TACAATCCCCCAACCCTTCTCCTG
	C808	AACGGGGTCTGATTGCTGGTAA
	C511	TCTGCATTTTCCCGCTCACTTTCA
	C1488	CCCGGGGGAACAAAGTCGTCAATA
	C268	AGCGGGCACCCTAAATACCACTTCA
	C1266	AATCCCCCAACCCTTCTCCTACCA

the distribution was statistically significant, the strength of the association between PMWS and individual virus was determined by calculating odds ratio.

RESULTS

The significance of PCV2 and several other viral pathogens (i.e., PRRSV, PPV, PEV, SIV, TGEV, PRCV, PLHV-1, PERV, BVDV) relative to development of PMWS was assessed using a case-control study design. The proportion of animals in each group, which were positive for an individual virus, is illustrated in Figure 1. Regardless of an individual animal's clinical status, all animals tested (N=93) were positive by RT-PCR for PERV, demonstrating that endogenous retrovirus is ubiquitous in swine. Besides retrovirus, PCV2 was the next most common viral agent detected in this study. Using various virological assays (VI, IHC, PCR), 29 of 31 PMWS pigs (93.5%) were positive for PCV2, whereas the virus was found in 35 of 56 control pigs (64%). Interestingly, PCV2 was not detected by any virological means in 2 of the 31 pigs affected by PMWS. On the other hand, these 2 pigs were positive for PRRSV, PLHV-1, and PERV. Fifty eight percent of PMWS and 18% and control pigs, were considered to be viremic at the time of sampling as PCV2 DNA was detected in their serum; however, no infectious virus was isolated from any of these pigs except one PMWS pig (Figure 2). Serologically all these animals had relatively high titers of IFA antibodies to PCV2, which failure of isolating PCV2 from sera may be attributed to. It is also worthwhile to note that PCV1, nonpathogenic strain, was also detected but only in control pigs (2%). In addition to PCV2, other viruses except TGEV and BVDV were detected in varying proportions of animals

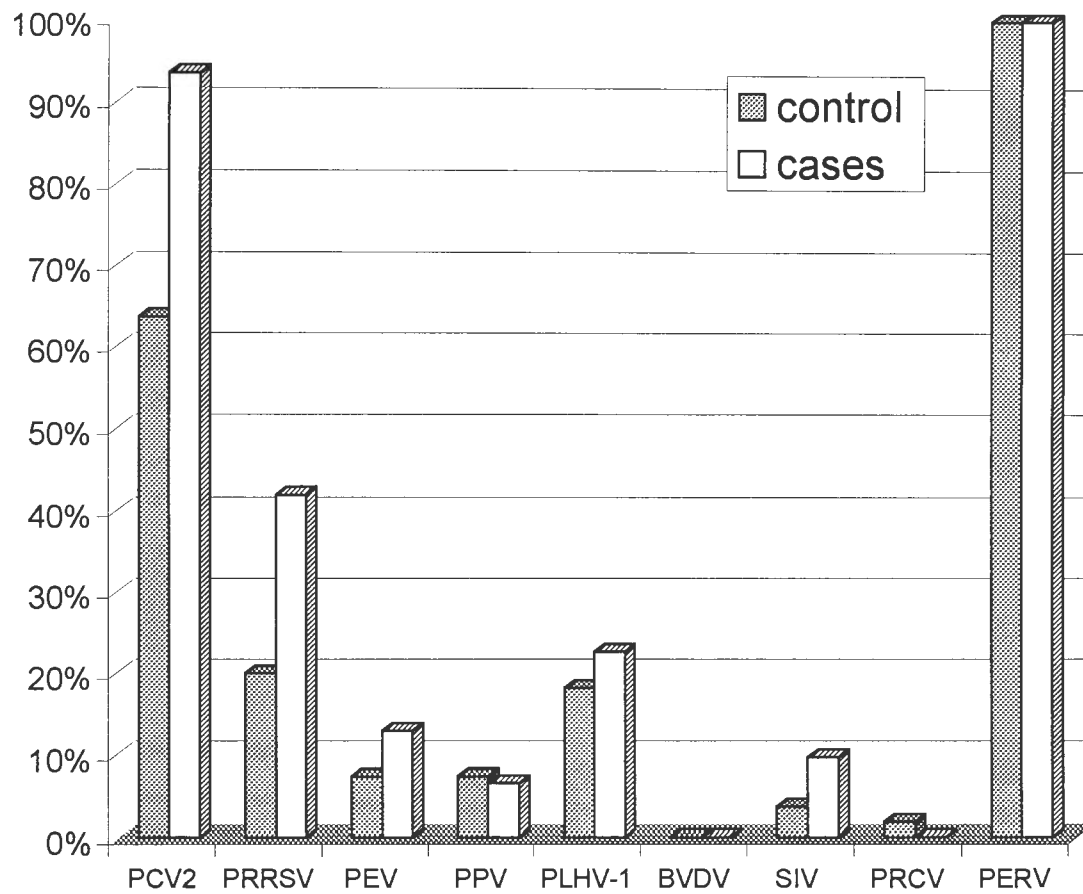


Figure 1. Proportion of animals positive for a given virus. For each virus, solid and shaded bars represent PMWS pigs and control pigs, respectively.

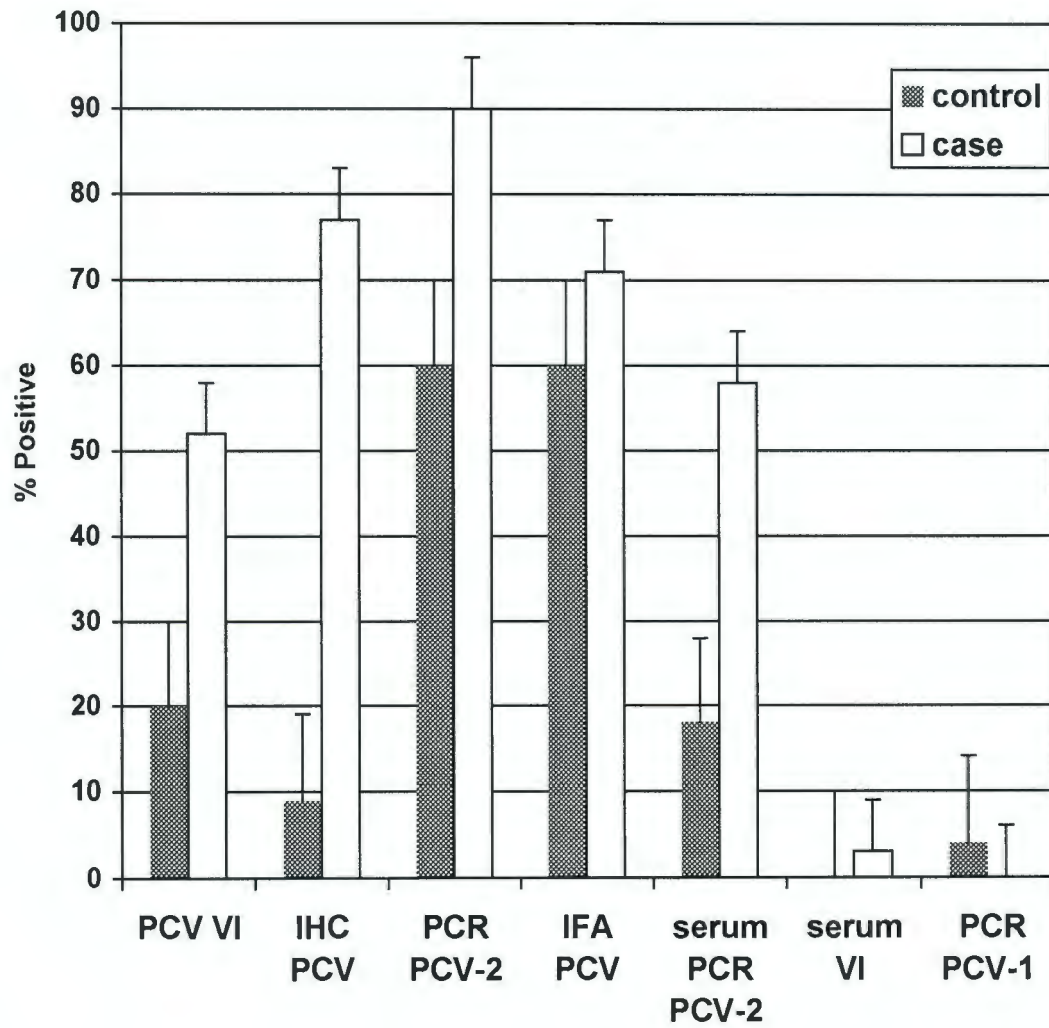


Figure 2. Performance of PCV-specific assays in detecting PCV2 in PMWS and control pigs. Bars represent proportion of samples positive for PCV2 by each test. Lines represent 95% confidence interval of each estimate.

examined (Figure 1). However, animals positive for these viruses except PRRSV were almost equally distributed between case and control pigs, suggesting that infection of PPV, PEV, SIV, PRCV, or PLHV-1 is virtually insignificant relative to PMWS ($p>0.05$). In contrast, PRRSV was present in 42% and 20% of PMWS and control animals, respectively, suggesting that the virus has some association with PMWS. Statistically, PCV2 as single entity was the most strongly associated with PMWS among viruses tested and the odd ratio was 9.3 (95% CI = 1.9, 45.3). That is, animals with PCV2 have 9.3 times higher risk for PMWS as compared to animals without PCV2. In comparison, pigs infected with PRRSV had odd ratio of 3.4 ($1.2 \leq 95\%CI \leq 9.8$) developing PMWS, which was considered significant ($p=0.027$) as compared to pigs without PRRSV infection. However, the risk for PMWS was much higher in pigs infected with both PCV2 and PRRSV (odd ratio = 31.2, $4.1 \leq 95\%CI \leq 238$, $p=0.009$). Such a significantly increased risk for PMWS was not observed in combination of PCV2 and other viruses or in combination of other viruses each other ($p>0.3$).

Genetic analysis of selected PCV2 from case and control pigs is summarized in Figure 3. Sequence homology among PCV2 from the same group was 98.9% for cases and 99.1 % for controls. Comparison of linear nucleotide sequence did not reveal significant difference in genomic sequences between PCV2 from clinical cases of PMWS and these ones from control pigs. Overall identity of nucleotide sequences among all PCV2 isolates compared was 99%. Virtually, sequence

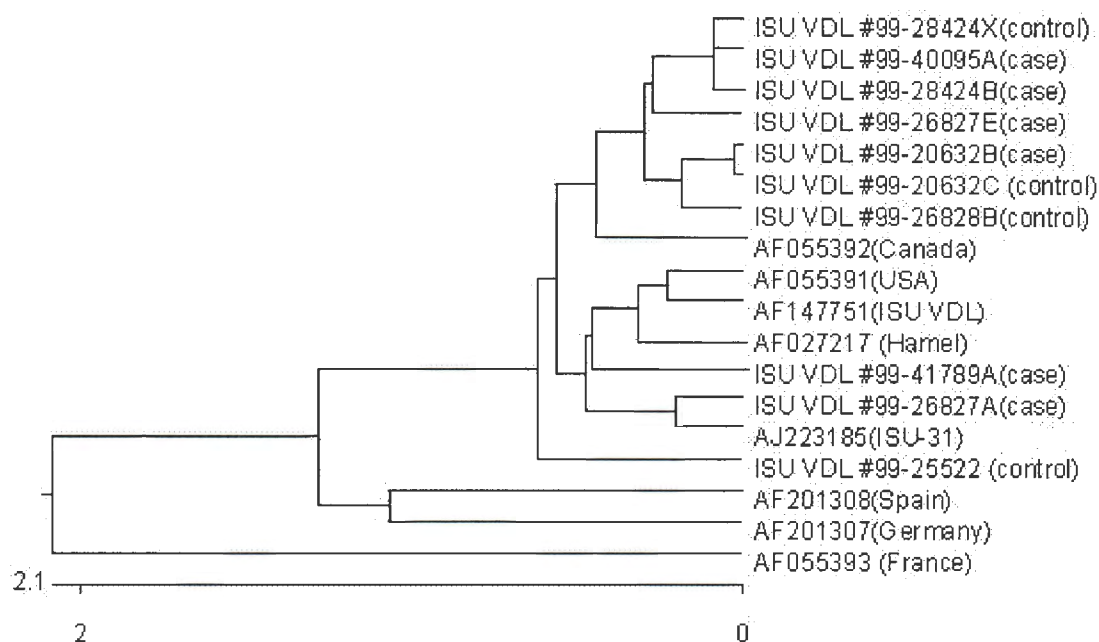


Figure 3. Phylogenetic diagram of PCV2 isolates from case and control group. The diagram is based on entire genome sequence using Clustal method for alignment.

motifs of non-coding region for virus replication (27) remained unchanged in all 10 isolates. Divergence in coding regions between PCV2 from cases and from controls was 0.7% at the nucleotide level and 2.4% at the deduced amino acid level. A phylogenetic analysis also did not demonstrate significant genetic differences between PCV2 from control pigs in this study and PCV2 isolates from clinical PMWS cases previously reported by other investigators. However, it is worthwhile to note that genetic distance was more close each other among North American PCV2 than to European ones.

Diagnostic performance of several laboratory assays (VI, IHC, PCR, IFA) developed to detect PCV2 or its infection were evaluated and summarized in Figures 4. Using disease status of animals (i.e., PMWS versus non-PMWS) as reference test, IHC showed the best overall performance in detecting PCV2 in association with PMWS. The diagnostic sensitivity and diagnostic specificity of IHC was estimated to be 80% and 91%, respectively. In contrast, PCR had the sensitivity of 90% but showed poor specificity (39%) with respect to detecting PMWS pigs. However, PCR results were in better agreement with serological results ($\kappa = 0.516$) as compared to degree of agreement between IHC and IFA ($\kappa = 0.11$), suggesting that PCR-based assay performs better than other tests in detecting PCV2 in animals regardless of their clinical status related to PMWS (Figure 4). For detecting PCV2 or viral antigens, tissues, particularly secondary lymphoid tissues were better than serum.

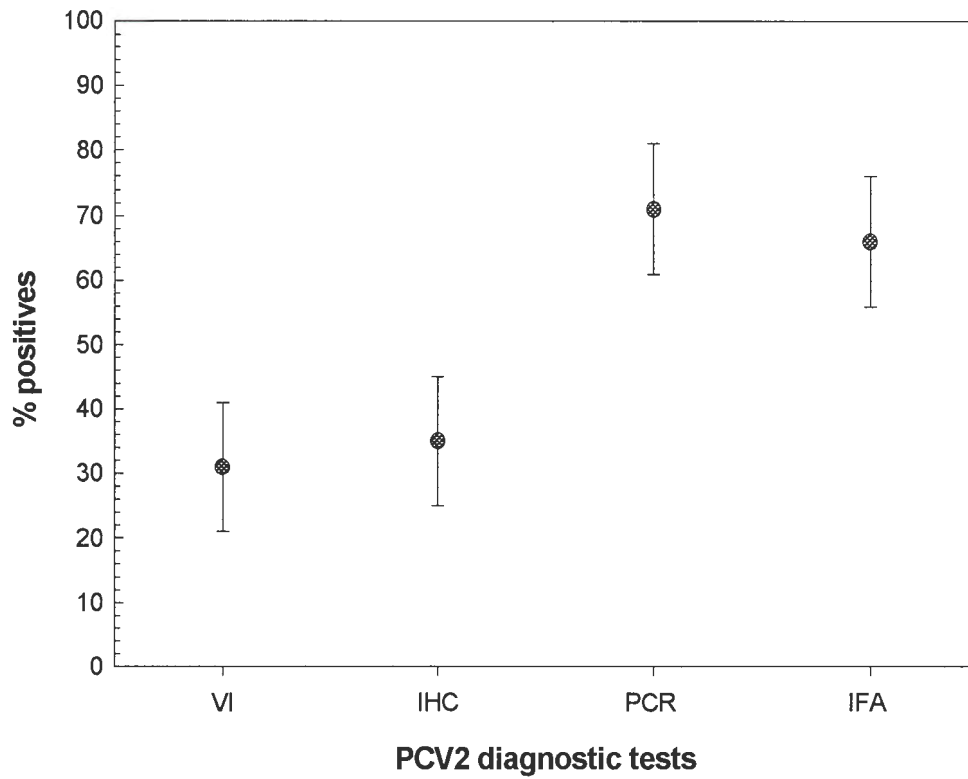


Figure 4. Comparison of PCV diagnostic tests in detecting PCV2 in clinical specimens regardless of disease status. Dots represent proportion of samples positive for PCV2 by each test. Lines represent 95% confidence interval of each estimate.

DISCUSSION

Porcine circovirus type2 is a newly recognized virus in the swine population and has been isolated and/or detected from clinical specimens of pigs diagnosed with PMWS (4;14;26;32). Despite diagnostic information strongly suggest that PCV2 is the causative agent of PMWS, experimental animal trials with PCV2 only, including the previous study in our laboratory have reproduced at best mild microscopic changes somewhat compatible with ones described in pigs affected with PMWS (3;18;23;34). Nevertheless no “wasting syndrome” has been reproduced even in immunocompromised animals. It raised the question of whether other factors besides PCV2 should be involved for PMWS if PCV2 plays primary role in PMWS. Concurrent infections with other agents, suboptimal management, and environmental stress have been suggested as potential co-factors. Experimentally, histopathological lesions typically observed in pigs affected by PMWS were reproduced in pigs accidentally or intentionally infected concurrently with PCV2 and porcine parvovirus (3;23;29). However all these animal trials failed to reproduce the disease. Therefore, the main objective of the case-control study was to assess the role of PCV2 alone and in conjunction with other major swine viral agents in PMWS in growing pigs in Iowa.

Our case-control study revealed that PCV2 as single entity had stronger association with PMWS (odd ratio = 9.3, $1.9 \leq 95\%CI \leq 45.3$) than other viral agents tested, which is in general agreement with previous reports by other investigators (16;37). However, PCV2 was also found in approximately 63% of pigs clinically normal or with clinical signs and pathological findings irrelevant to PMWS.

Furthermore, the virus was not detected in 2 of the 31 pigs (6.5%) that did not thrive well and had histopathological lesions typical of PMWS. These observations raised the question of why the majority of animals proven to be infected with PCV2 were clinically and pathologically silent related to PMWS. Furthermore, although the risk for PMWS was shown to be higher in pigs infected with PCV2 than pigs without PCV2 infection, the estimated odd ratio of 9.3 (95% CI = 1.9, 45.3) is relatively low, i.e., strength of association is weak, if PCV2 infection is considered to be the causality of PMWS. In comparison, PRRSV infection showed odd ratio of 11.2 when PRRS emerged as mystery swine disease (48). Consequently, our data suggest that other factors besides PCV2 may be required for development of PMWS in pigs if PCV2 can predispose pigs to PMWS. One of several possible co-factors is synergistic effect of other viral agents. In this regard, several major swine viral pathogens, such as PRRSV, parvovirus, influenza virus, coronavirus and enterovirus, were examined in this study. Apparently, the risk for PMWS was dramatically increased if animal was co-infected with PRRSV (odd ratio =31.2, $4.1 \leq 95\%CI \leq 238$), suggesting that concurrent infection of PRRSV contributes to development of PMWS in pigs infected with PCV2. Experimentally, it was reported that cesarean-derived colostrum-deprived animals were icteric, wasted, and died of increased severity of disease when infected with PCV2 and PRRSV (20). A higher risk for PMWS by concurrent infection of PCV2 and PRRSV is not unexpected result since PRRSV is known to be prevalent in Iowa swine. Practically, this observation suggests that the risk for PMWS and the severity of disease could be reduced by better control of PRRSV. Nevertheless, it remains to be answered why

13% of control pigs that were infected concurrently with PCV2 and PRRSV did not develop PMWS if co-infection with PRRSV would be critical for PMWS.

Such an increasing risk for PMWS was not observed with other swine viral pathogens such as parvovirus, enteroviruses, influenza virus, and coronaviruses ($p>0.3$). In particular, it was in contrast to previous reports (16) that no significant potentiation for PMWS was observed in pigs concurrently infected with PCV2 and PPV. In our study, very few pigs were determined to have parvovirus at the time of sample collection. It may have been attributed to the fact that our study used conventionally raised pigs which are considered to be immunologically competent and most of which had PPV antibodies that appeared to be of colostrum origin. If any of the viruses described above has any positive effect on development of PMWS, the order and timing of infection would attribute to the difference in outcome, which remains to be further studied. Besides common swine viral pathogens, viral agents that are capable of affecting the immune system of pigs were also evaluated in the present study, including retrovirus, herpesvirus, and BVDV (1;13;44). These viruses are known to replicate in porcine cells of the immune system and dysfunction or suppress the immunity or lymphoid proliferation of the pigs. Such a condition was postulated to be required for extensive replication of PCV2 in pigs (3). In our study, all pigs examined were positive for nucleic acid of porcine endogenous retrovirus (PERV), indicating that endogenous retrovirus is ubiquitous in swine. Since its distribution between PMWS and control pigs was even, PERV would not be considered to contribute to PMWS. Porcine lymphotropic herpesvirus type 1 is a gamma herpesvirus recently identified in pigs (13). The virus

was speculated to be avirulent to swine (13). In agreement with previous report, our study did not reveal the significant role of PLHV-1 in development of PMWS.

However, it is worthwhile to note that the virus appears to be widespread in U.S.

domestic swine as detected in 41% of pigs tested in our study. PLHV-1 is

considered to pose the potential risk for disease in immunocompromised human

receiving xenotransplant of pig origin. Bovine viral diarrhea virus is known to

replicate in and destroy subpopulation of lymphocytes in the ruminant (21;33) and in

pigs (44). Recently it was reported that BVDV infection can enhance the virulence of

other viruses like TGEV in pigs with very similar lesions reported for PMWS:

generalized lymphocyte depletion throughout the lymphatic system and villous

atrophy in the intestinal tract (40;44). It has also been reported that contamination

of cell cultures with BVDV through fetal bovine serum is common (8). Consequently,

it is possible that some of attenuated or modified lived virus vaccines may contain

BVDV. Despite the strong possibility that BVDV can be present in pigs, none of

pigs tested in our study had virological or serological evidence of BVDV infection,

demonstrating no association with PMWS. Another factor possibly contributing to

different outcome of PCV2 infection is the variability in the pathogenicity among

PCV2. Such a possibility was evaluated indirectly using sequence analysis of the

entire genome of the virus. Based on linear nucleotide sequence, no significant

genetic differences were observed among PCV2 from PMWS and clinically normal

pigs (Figure 3). Overall sequence homology among PCV2 regardless of their

source was approximately 99%. Furthermore, no genetic change/marker that is

unique to PCV2 from the same clinical status group (i.e., PMWS versus control was

observed either. Since virus in clinical specimens were directly compared by PCR amplification and sequencing to avoid any selective pressure during *in-vitro* cell culture, our observations indicate that apparent differences in virulence, if any, may not be associated with genetic traits of the virus. However, potential differences in the pathogenicity needs to be studied using a biological system, i.e., animal trial, prior to coming to the conclusion on this. In addition, the difference among PCV2 should be investigated at the gene expression level. All laboratory procedures developed for detecting PCV2 or its infection, such as virus isolation, PCR, IHC and serology, are known to be specific (5;15;24;28;34;39;41). All tests are also considered to have reasonable sensitivity for detecting PCV2 in clinical cases; however, there has been no report comparing the diagnostic performance of available PCV2 tests. In this study, several laboratory procedures for PCV diagnosis were compared. Using disease status of animals (i.e., PMWS versus non-PMWS) as reference test, IHC for PCV showed a good predictive value for diagnosing PMWS. Virus isolation results were in a good agreement with IHC results ($\kappa = 0.445$). Interestingly, PCR showed diagnostic sensitivity and diagnostic specificity of 90% and 39%, respectively, in conjunction with diagnosing PMWS pigs, suggesting that PCR may result in a lot of “false” positives. However, the trend of higher PCV2 positive rate by PCR was the same in both PMWS and control pigs (Figure 2). In addition, PCR results were highly correlated with IFA results ($\kappa = 0.516$) as compared to the degree of agreement between IHC and IFA ($\kappa = 0.11$). These observations indicate that PCR test should be considered the most sensitive test among laboratory tests for detecting PCV2 as

illustrated in Figure 4. Yet, positive PCR results for PCV2 may need careful interpretation because of its apparently low predictive value for PMWS. Since our investigation was an observational study, causality of disease could not be determined. Yet, a case-control study is an easy and fast approach to determine the strength of association that a target organism has with the problem of concern. Based on difference in percent positives between cases and controls, PCV2 tends to be highly associated with PMWS as compared to other viral agents evaluated in this study. At the same time, PCV2 appears to be highly prevalent in swine regardless of clinical status of animals as more than 60% of animals without apparent clinical signs and pathological changes related to PMWS harbor the virus. At the infectious disease point of view, these observations suggest that PCV2 may be a bystander of PMWS. Such an observation raises then the question of what makes the difference in outcome of infection if PCV2 should be considered as the primary cause of PMWS. Apparently, such a difference was not attributed to genotype of PCV2 since no significant genetic difference was found among PCV2 from PMWS and clinical normal pigs. Although concurrent infection with PRRSV increased the risk for PMWS dramatically, other viral agents whose infection may have similar effect on pigs failed to demonstrate the same effect. Consequently, it is hard to make an absolute connection between PCV2 infection alone or concurrent infections and development of PMWS. Collectively, the causality of PMWS remains to be determined. Furthermore, the actual role and pathogenesis of PCV2 in PMWS remains to be re-evaluated.

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GENERAL DISCUSSION

Introduction

Porcine circovirus that is genetically (59) and antigenically (1) distinct from PCV found in PK-15 cell line (PCV1), has been frequently isolated from or detected in growing pigs affected by severe “wasting” syndrome which led to the death of affected animals in so-called “high-health” status herds. The syndrome was named Postweaning Multisystemic Wasting Syndrome (PMWS) after its characteristic clinical manifestation. To reflect the difference between two porcine circoviruses, isolates from PMWS case were designated PCV2 and PK-15 contaminant PCV1. Although diagnostic information highly suggested that PCV2 is associated with PMWS (4;22;33), some experimental animal trials failed to reproduce the disease or pathological changes in pigs inoculated with PCV2 (42;75). That is, Koch’s postulates have not been fulfilled. The purpose of this study was to characterize the response of pigs to PCV2 infection and evaluate the role of porcine circovirus in PMWS.

PCV2 infection in pigs

The preceding study demonstrated that naïve young pigs are susceptible to PCV2. Inoculated pigs became viremic and developed a virus-specific antibody response. Furthermore, viral DNA and antigens were detected in tissues. However, no clinical signs or gross pathological changes were observed in any of inoculated pigs during the study but only very mild histopathological changes,

suggesting that PCV2 or the isolate used may not be pathogenic for swine or additional condition(s) may be required for PMWS. A case-control study was conducted to clarify such inconclusive observations from the animal trial.

Statistically, PCV2 was demonstrated to be highly associated with PMWS (odd ratio = 9.3, $p=0.005$) as compared to other major swine viral pathogens tested for. Risk for PMWS was much higher if an animal was co-infected with porcine reproductive and respiratory syndrome virus (odd ratio =31.2, $p=0.0009$). However, PCV2 was also found in a high proportion of clinically unaffected animals and was not detected in a few of pigs affected by PMWS. Furthermore, no significant genetic differences were observed between PCV2 isolates from PMWS and clinically normal pigs, suggesting that differences in pathogenicity among PCV2 isolates, if any, should not be attributed to genetic traits of the virus. Collectively, the causal role of PCV2 in PMWS is in question. If PCV2 should have a causal role in PMWS, the presence of additional factor(s) or condition(s) that were not be assessed in this study may be required to induce PMWS, which remains to be clarified.

Causes of “wasting” disease and lymphoid depletion

Progressive weight loss is cardinal clinical sign observed in pigs affected by PMWS. Microscopically, lymphoid depletion and granulomatous inflammation are known to be characteristic lesions in PMWS pigs. However, other conditions have been known to cause similar changes in pigs as described for PMWS, which need to be taken into consideration in any future studies. For example, lymphoid depletion can be induced by infections with *Toxoplasma spp.* (10), Tchothencene

toxin T-2 (110), hog cholera (93), African swine fever (76), steroids (89) and BVD (112).

“Wasting” disease can be caused by many pathogens, such as PRRSV, hemagglutinating encephalomyelitis virus, influenza virus, *Lawsonia intracellularis*, *Mycoplasma hyopneumonia*, *Haemophilus parasuis*, *Mycobacterium avium-intracellulare*, Colibacteriosis, and Cryptosporidiosis (33). In addition, any conditions that interfere with normal development of thymus during the fetal stage may lead animals to gradual weight loss after birth as soon as weaned. This phenomenon has been experimentally demonstrated in dogs, kittens and mice (20;84;85).

Granulomatous inflammation is a general description of the lesions by giant cell formation that can be caused by many pathogens and non-pathogens during chronic immune reaction due to disorders in function of macrophages (16). When phagocytes such as activated macrophages, are unable to “clear” ingested antigens due to their poor degradable nature, the cells accumulate in the tissue and develop in multinucleated giant cells, which will form into granulomas (89). Activated macrophages in granulomas produce cytokines such as TNF- α , IL-1, IL-6, IFN- α , which may lead to the wasting disease (28;32). It is possible that the granulomatous reactions are secondary to tissue damage initiated by another mechanism.

Role of PCV2 protein in immune response

Although questions remain as to the pathogenesis of PMWS, this study contributed to our basic understanding of virus structure and host response. The

protein composition of PCV2 is unknown, but based on an analysis of the viral genome, PCV2 has been postulated to contain as many as 11 open reading frames (30). However, western immunoblot analysis in this study demonstrated only 3 viral polypeptides with molecular masses of 28, 28.5, and 35kD. Of these, the 28 and 35kD proteins matched the expected molecular weight of the polypeptides predicted to be encoded by ORFs 1 and 2 (30;59;60) and, on that basis, may be postulated to be the protein products of ORF 2 and 1, respectively.

The 28kD protein was the most immunogenic of the 3 proteins identified, based on the observation that antibodies specific for this protein appeared first and persisted to the end of the study. Furthermore, antibody against the 28kD protein was not detected using PCV1 antigens on western immunoblotting. These results suggest that the 28kD protein may provide the antigenic basis for the development of diagnostic tests for the detection of PCV2-specific antibody.

Results of western immunoblot analysis and serological monitoring data suggested that the 28.5kD and/or 35kD proteins were associated with the induction of neutralizing antibody and the 28kD protein was not. Although it is not certain whether the 28.5kD protein, the 35kD protein, or both are responsible for virus neutralization, neutralizing activity in sera appeared to correlate with the appearance of antibodies to the 35kD protein. Additional research is necessary to define the role of antibodies specific for 28.5kD and 35kD protein in viral neutralization.

Although significant genetic and antigenic differences exist between PCV1 and PCV2, some degree of cross reactivity between PCV1 and PCV2 on IFA test

has been demonstrated in this study as well as by other investigators (6;48). At the protein level, our study suggest that antibody to the 35kD protein is responsible for the cross-reaction. This conclusion is based on the fact that antibody from pigs infected with PCV2 recognized this protein in both types of PCV on western immunoblotting. Assuming that the 35kD protein is the protein product of ORF1, our conclusion is also supported by sequence analysis data demonstrating high DNA homology in ORF1 between PCV1 and PCV2 (30;48;59;60). Previous serological surveys using PCV1 demonstrated that PCV infection was common in swine. Data from the present study showed that earlier serological surveys would have detected antibodies against either PCV1 or PCV2. That being the case, PCV2 may have been present in the swine population for quite some time and not a recent introduction. This conclusion is supported by a recent serological survey demonstrating the presence of PCV2 as early as in 1973 (111).

Future study

This study demonstrated that PCV2 is infectious for pigs. Immune ontogeny with role of PCV2 protein in immune response was characterized. However, the causal role of PCV2 in PMWS could not be conclusively demonstrated, although the virus appears to be a necessary component for PMWS. Therefore, the pathogenesis of PCV2 and PMWS remains to be further studied. If PCV2 should have a causal role in PMWS, all necessary co-factors should be identified for developing prevention and control strategies for the syndrome.

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